Journal of
DAIRY SCIENCE

Vol. 43 February, 1960 Number 2

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1934	October	1957	February, June, October
		1958	March, June
		1959	January

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PEOPLE AND EVENTS

Richard J. Werner, Milk Industry Foundation Head, To Retire

RICHARD J. WERNER, executive director of the Milk Industry Foundation, will retire in 1960. Mr. Werner, who has headed this international organization of milk processors and distributors for the last 6 yr., reached the retirement age on December 3rd. However, by request of the executive committee of the Foundation, he will not retire until September 30, 1960, being on leave after June 30.

R. J. Werner

Born in Minnesota in 1894, Mr. Werner is a veteran of many years of distinguished service to the dairy industry. His early boyhood was spent on a dairy farm in the San Joaquin Valley of California. He worked his way through Oregon State College and earned a Bachelor of Science degree in 1917. He served as the first county agricultural agent for Clackamas County, Oregon.

After serving in the U. S. Army during World War I, he returned to California to manage a dairy farm. Later, he became an employee of the Golden State Dairy Products Company and attained the position of field manager. In 1927, Mr. Werner was appointed California State Supervisor of Agricultural Education, a responsible position in the school system. Later, he was promoted to Commissioner of Secondary Education for California. From this position, he moved to the presidency of Hartnell College in Salinas, California.

Mr. Werner again volunteered for military service during World War II, attaining the rank of colonel. He earned seven battle stars for participation in the European Theatre, and received the Bronze Star, the Legion of Merit, the Purple Heart, and received the Croix de Guerre from both France and Belgium. He is a Commander of the Order of the Crown of Italy and a Commander of the Order of Orange Nassau of the Netherlands.

Returning from the war in 1945, he became manager of the California Dairy Industry Advisory Board. In 1951, he moved to Washington to become assistant executive director of the Milk Industry Foundation, and 2 yr. later was promoted to executive director.

Under his leadership, the Milk Industry Foundation has grown to 1,600 members, with over a hundred members in Canada, and more than 20 in ten foreign countries. He also has expanded the membership services of the organization, and conducted a personal campaign to encourage more widespread promotion of cottage cheese and sour cream.

Besides the degree of Bachelor of Science, Mr. Werner holds a Master of Science degree from the University of California and an honorary Doctor of Science degree from the University of Naples in Italy.

Mr. Werner is active in civic and charitable organizations. He is a past-president of the Salinas, California Rotary Club, a former member of the San Francisco Rotary, and is an active member of the Washington, D. C. Rotary. He is married to the former Gay Martin of Palo Alto, California. They live in Washington, D.C.

COLLEGE AND UNIVERSITY NEWS

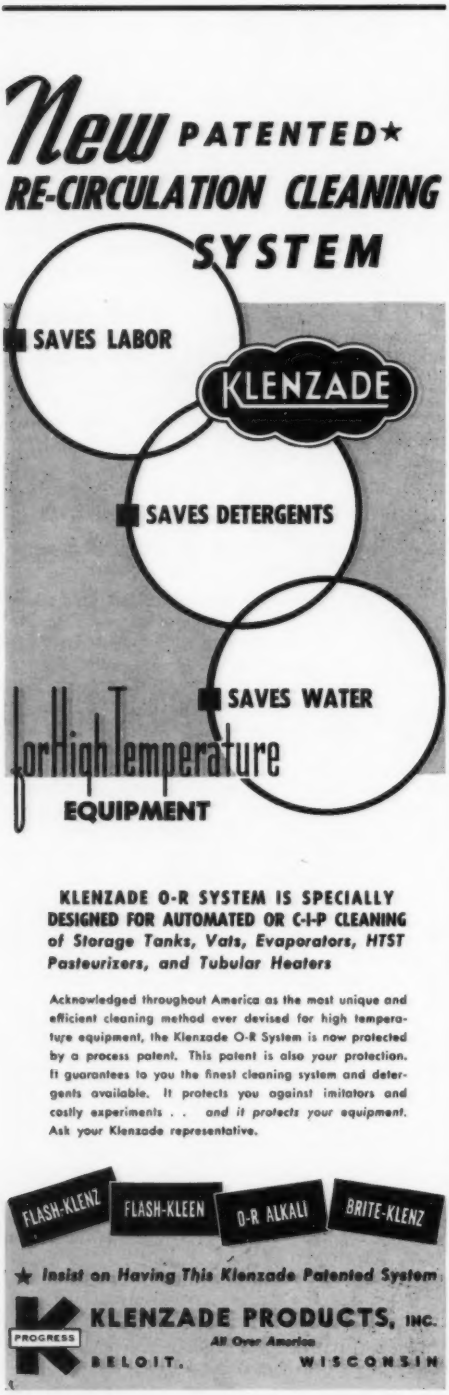
Lynn Brown Appointed in Dairy Extension at University of Connecticut

LYNN BROWN has been appointed Extension Dairyman in the Department of Animal Industries at the University of Connecticut. He assumed his new duties on January 4. Dr. Brown was born on an Iowa dairy farm and participated for 8 yr. in 4-H dairy work. He attended Iowa State College and received his B.S. in 1950. He taught in the veterans' agricultural program for 1 yr., then spend 2 yr. in the Army. He then was a vocational agricultural teacher for 2 yr., previous to initiating his graduate work. He completed his Ph.D. degree in June of this year and since that time has been in charge of the Dairy Nutrition Laboratory at Iowa State. His major in graduate school was dairy nutrition and management and his research was on the cause and prevention of bloat. He has also done research on milk replacers in calf feeding.

Dr. Brown's areas of concentration in Dairy Extension at Connecticut will be dairy nutrition and feeding programs and the 4-H dairy work. Dr. Brown also will be associated with Dr. Bob Benson in the dairy management and testing programs. He and Mrs. Brown will reside in Storrs with their two children.

ROBERT G. JENSEN, Assistant Professor of Dairy Manufacturing, has received a research grant of $34,475 from the Public Health Service to study the Fatty Acid Content of Milk Lipids.

GEORGE W. GANDER is a co-investigator in the project, which extends for 2 yr.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Arizona State

The Ninth Annual Dairymen's Conference will be held at Arizona State University on April 12, 1960. Among those who will participate in discussing the theme of Stress in Dairy Cattle will be L. W. HOLM of the University of California; V. R. SMITH, University of Arizona; R. F. FREEMAN, Arizona State University; B. P. CARDON, Arizona Flour Mills, Tucson, Arizona; and W. E. WEAVER, Dairyman, Tolleson, Arizona.

Florida News

An item in the November JOURNAL, mentioning that D. H. KLEYN had been named assistant professor of dairy manufacturing and assistant dairy technologist at the University of Florida, was in error in stating that Mr. Kleyn had received his M.S. degree from Ohio State University in 1953.

Mr. Kleyn received his B.S. degree in dairy technology from Ohio State in 1953 and, after serving in the U. S. Navy from June, 1953, to October, 1954, received his M.S. degree from Cornell University in 1956. Before becoming a faculty member at Florida, he had completed all of the requirements for the Ph.D. degree, which he will receive in February.

Cassius Clay Hayden
1871-1959

Hayden Hall became the official designation of the building housing the dairy science offices and laboratories at the Ohio Agricultural Experiment Station at Wooster by action of the Board of Control on December 11, 1959. This act honors the dairy scientist who was the first chief of the department, then called Dairy Industry, who guided the research program and service for the dairy industry of Ohio from 1912—1946.

C. C. Hayden

CASSIUS CLAY HAYDEN was born in Hemlock, Ohio, December 1, 1871. For 19 yr. he lived in one or another of three of Ohio's southern hill counties. At this age, not yet having finished with the eighth grade, his ambition for formal schooling took him to Spring Arbor Seminary in Michigan, where he completed the equivalent of a high-school course in 4 yr. Returning to southern Ohio, he taught school for a year before entering The Ohio State University, where he enrolled in the agriculture course. He received his Bachelor of Science degree in 1901 and, after 6 mo. of working in a creamery near Springfield, Ohio, went to the University of Wisconsin for graduate study. Shortly

thereafter, he accepted a joint assistantship at the University of Illinois, in the college and experiment station. He remained at this institution for 8½ yr., during which period he obtained the Master's Degree (1909). He advanced to the position of Assistant Chief of the Dairy Department, a rank he held from 1903 to 1912.

Mr. Hayden assumed the duties of chief of the newly established Dairy Industry Department in 1912, succeeding R. E. Caldwell who had been assistant in charge of dairying within the Animal Industries Department. His only professional associate was Chemist A. E. Perkins. Under Hayden's direction, the department grew in numbers of professional staff until the time of his retirement, when it numbered six full-time staff members, with graduate students and laboratory technicians all in some phase of nutritional work.

One of Chief Hayden's first major responsibilities was to design and help construct a building to replace the creamery which burned in November, 1913. Hardly had the ashes cooled before he was in consultation with the state architect with plans for a much expanded building to house both offices and laboratories. Not being satisfied with the progress of the craftsmen on the job, the Chief himself helped lay the brick with mortar.

Studies in contrast are of interest to us as a diadem against which to portray the life of a colleague. Against our modern industry, shaped by the findings of recent research, engineering, and regulations, are the following descriptive notes chronicled for the author by A. E. Perkins, life-time professional colleague of the subject of this biography. They characterize the period during which Mr. Hayden came to Ohio.

Many of the cattle were removed from the herd because of tuberculosis infection. Brucellosis later was to be a principal health deterrent. Raw milk was sold at the station for 5c a quart. Professional staff were paid $1,200 annually, with a 2-wk. vacation from which observed holidays were deducted. Barn help started at 15¢ an hour and for faithful service could hope eventually to rise to 18¢. The dairy office was on the second floor of a residence, with the nutrition laboratory being a remodelled room in the creamery previously used for the storage of ice. One of the main summer activities was preparing and displaying exhibits at county and state fairs, the importance of which is portrayed by Nevens.[1] Mr. Hayden, with other well-established senior station staff members, rode or drove horses to and from work for the period up to 1926, after which he walked, until 1937, at which time he purchased his first automobile. He, with other dairy staff members and a male secretary, assisted the herdsman in manning the dairy herd during periods of labor shortage during World War I.

From the standpoint of the American Dairy Science Association, it is significant to record that C. C. Hayden was a charter member along with 17 other pioneers.[2] The memory of this participation was one that he always cherished. He was chairman of the production section in 1926 and elected honorary life member in 1944.

His personal investigations centered largely on silage preservatives and the value of ensiling various farm crops. His first publication for which a reference was found (1912) was Feeding Dairy Cows. This original circular underwent many revisions to keep up-to-date information before dairymen. His name is also recorded with the committee on Feeding Standards for milk production as early as 1919. His ideas on measuring the breeding value of cow families were well detailed, but too far ahead of his day to be considered useful. He realized some of his objectives in this area through his favorite project, on proving bulls selected as calves according to the best known standards. Summaries of this investigation constituted the subject of his last major research publication in 1946. All together, 56 papers dealing with some phase of dairy science have been catalogued.

Administratively, his services to Ohio included a profound interest in getting people to become active in the eradication and control of tuberculosis and brucellosis, adequate tests for creamery butter, and disseminating ideas and facts on feeding and management to Ohio dairymen.

Personally, Mr. Hayden was known as a man of sterling traits, dry humor, realistic approach to problems, and close adherence to his established standards. He believed in punctuality in work, devotion to duty, dedication to ideals. Those traits he possessed, and he expected to find them in others whom he chose to become associated with him.

Surviving Mr. Hayden at the time of his death on November 18, 1959, were his three sons, Dr. Edgar C. Hayden of the University of Illinois Physics Department, John O. Hayden with the Western Electric Co. of Lynchburg, Virginia, and Dr. Richard A. Hayden of the Georgia Agricultural Experiment Station Horticulture Department. Also surviving him are two brothers and a sister. Mrs. Hayden died in 1957.

[1] NEVENS, W. B. The Preparation of a Dairy Exhibit. J. Dairy Sci., 2: 415. 1919.

[2] TROUT, G. M. Fifty Years of the American Dairy Science Association. J. Dairy Sci., 39: 625. 1956.

News from Ohio State

Officers of the Ohio Dairy Products Association for 1960 have just been announced, and are as follows:

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Other members of the board of trustees for 1960 will be: Milk Distributors: E. C. GREINER, Greiner Dairy Company, Zanesville, president; O. A. DEAN, Dean Dairy Company, Cleveland, vice-president; E. MOSER, Med-O-Pure Dairy, Washington C.H., past-president. Milk Products Manufacturers: J. MARABELLA, Carnation Company, Coshocton, president; R. J. GILBERT, Pet Milk Company, Bryan, vice-president; R. BUEHLER, M&R Dietetic Laboratories, Inc., Columbus, past-president. Ice Cream Manufacturers: R. L. BOYNTON, Harmony Farm, Columbus, president; O. G. HOFFMAN, Royal Crest, Dayton, vice-president; ROGER UMBS, San-A-Pure Dairy, Findlay, past-president. Butter Manufacturers: E. GRIESER, Napoleon Creamery Company, Napoleon, president; J. A. HYEST, Cloverland Dairy, Flushing, vice-president; H. HAMILTON, Eldorado Creamery, Eldorado, past-president.

The January meetings of the four Ohio Dairy Technology Societies—Maumee Valley, Northeastern Ohio, Central Ohio, and Cincinnati—emphasized cottage cheese. R. L. ZIEGLER, Ziegler and Son, Topeka, Kansas, conducted cottage cheese clinics and discussed factors that affect cottage cheese quality.

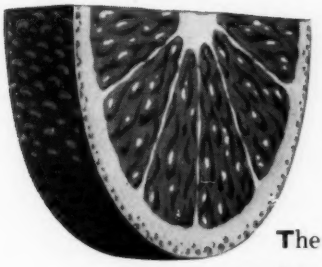
Oregon State

The Eighth Annual Oregon Milk Sanitarians short course was recently completed on the campus of Oregon State College. The short course is sponsored annually by the Oregon Department of Agriculture and Oregon State College. Presentation on farm-tank sediment testing, pre-plating incubation of milk samples, farm-tank cleaning, farm lay-outs, and the Q fever problems were made during the first day of the short course. The second day was devoted to an explanation and discussion of the revised Fluid Milk Regulations for the state of Oregon. Some 50 sanitarians, field men, and other dairy industry representatives were present for the two-day meeting.

ALVIN TESDALL of the Oregon Department of Agriculture was recently elected the president for the Oregon Association of Milk Sanitarians. He succeeds ERWIN LAUGHLIN of the Western Dairy Equipment Company. ART PARKER of the City of Portland Milk Inspection staff was elected vice-president.

E. A. DAY of Oregon State College recently received a 3-yr. grant from the National Institutes of Health, to expand his work on the basic principles of fat oxidation.

THIS IMPORTANT SLICE OF THE DAIRY BUSINESS CALLS FOR KRAFT QUALITY



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KRAFT

RICHARD J. WERNER, executive director of the Milk Industry Foundation, spent the first week of November in Oregon on a cottage cheese sales promotion program. A series of four meetings was held in various locations within the state. He also spoke to the 36 cottage cheese makers present at the cottage cheese clinic at Oregon State College on November 6. OSCAR HAGG, Extension Dairy Marketing Specialist, DONALD THOMAS, executive secretary of the Oregon Dairy Products Commission, and J. O. YOUNG, Assistant Professor of Dairy Technology, accompanied Werner on his tour throughout the state.

The Ice Cream Merchandising Institute, Inc. will present their Advanced Sales Training Course in Portland, Oregon, beginning the week of January 25, 1960. The Washington State Dairy Foundation and Oregon Dairy Industries are co-sponsoring the course with the Merchandising Institute.

BUSINESS AND INDUSTRY NEWS

Four Firms Licensed to Produce Instant Dry Milk Processing Equipment in U. S. and Abroad

Prospects for wide use of instant dry milk throughout the world appeared promising with the announcement from Dairy Foods, Incorporated, of Oakland, California, that two European and two American firms have been licensed to manufacture the special equipment required to produce the product.

The European companies are Lurgi Gesellschaft fur Warmetechnik M.b.h. of Frankfurt, Main, Germany, and Niro Atomizer, Ltd., of Copenhagen, Denmark.

The American firms are C. E. Rogers Company of Detroit, Michigan, and the Henszey Company of Watertown, Wisconsin. They will build and install equipment only for companies licensed to use Dairy Foods, Incorporated processes.

Dairy Foods, Incorporated, was organized in 1958 when Foremost Dairies and Carnation Company pooled basic patents covering instant whole milk, instant nonfat milk, instant flavored milk, and other related dry milk products.

The jointly owned company is licensing dairy and food companies on a nonexclusive basis to manufacture these products and to market them under their own respective brand names.

Beatrice Foods Honors Ten at Awards Luncheon

Ten members of the General Office staff of Beatrice Foods Company were honored on Dec. 11 at an awards luncheon at the Ambassador East Hotel.

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A Review of Research in Milk Production

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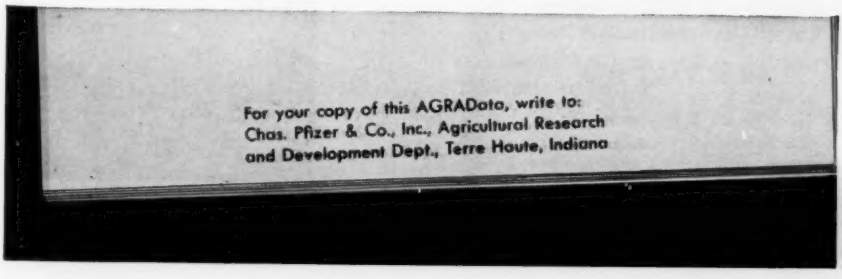
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The Creamery Package Mfg. Company
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Los Angeles, California

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I am enclosing a picture of our freezer room, taken after the installation of the Creamery Package 3M-15 Continuous Ice Cream Freezer.

It is putting it mildly to say we are highly pleased with the performance of the CP 3M-15. It has replaced five freezers formerly needed to do the same job. In operation for three months, it is saving $75 to $100 per shift through its accurate overrun control, easy operation and clean-up time savings.

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FRIGID PROCESS COMPANY

Jack Goodman, President

JG:ml

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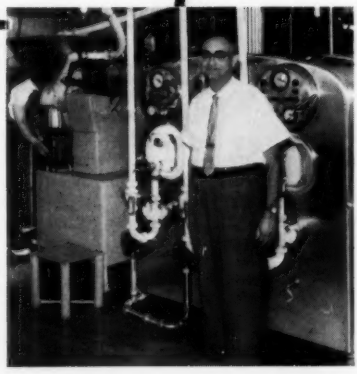

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Among those receiving the special service emblems from President WILLIAM G. KARNES were LEO J. HIMMELSBACH, 15330 Hastings Dr., Dolton, assistant general auditor, 30 yr. of service; ORVILLE H. KAHRL, 221 Union St., Joliet, assistant general engineer, 20 yr.; PAULINE PEARSON, 3121 N. Sheridan Rd., the company's home economist for 10 yr. and AUBREY LEGRANDE, 336 Indianwood Blvd., Park Forest, cream procurement specialist, 20 yr.

At Beatrice Foods Company Awards Luncheon. President W. G. Karnes (second from right) handing special service emblem to Pauline Pearson. Also receiving emblems were (left to right) L. J Himmelsbach, O. H. Kahrl, and Aubrey LeGrande.

Miss Pearson, the company's "Beatrice Cooke," taught home economics in a downstate high school and served as home economist for Wearever Aluminum Co. and International Harvester Co., and in the control laboratories of American Bakeries Corp., before joining Beatrice Foods as home economist.

Himmelsbach began his Beatrice Foods service as an office clerk, advancing through accounting and office management jobs to his present position as assistant general auditor.

Kahrl joined the company's maintenance department 20 yr. ago, working in the Beatrice Foods plants in Waukegan, Danville, and Kankakee before moving up in the general office to his present position.

LeGrande, a specialist in cream procurement for the dairy company, has served as cream procurement manager at Champaign, Ill., and Lincoln, Nebr.

Others (not shown) receiving the service emblems were DOROTHY J. MCINTYRE, 6050 N. Claremont Ave., credit clerk, 15 yr.; MARGARET B. REECE, 6114 N. Winthrop Ave., receptionist, 15 yr.; RALPH S. DEDRICK, 191 Ash Ave., Park Forest, cost accountant, 10 yr.; FLORENCE M. MONAGHAN, 6559 Lakewood Ave., secretary, 5 yr.; and KELLY M. GARLOCK, JR., 5057 Sheridan Rd., accountant, 5 yr.; JOAN D. WITKOWSKI, 2945 W. Fullerton, secretary, 5 yr.

Cherry-Burrell Notes

Cherry-Burrell Corporation has established a new subsidiary in Mexico City, it was recently announced by HOWARD H. CHERRY, JR., president. Prospect of a Latin American economic union, with Mexico as its industrial

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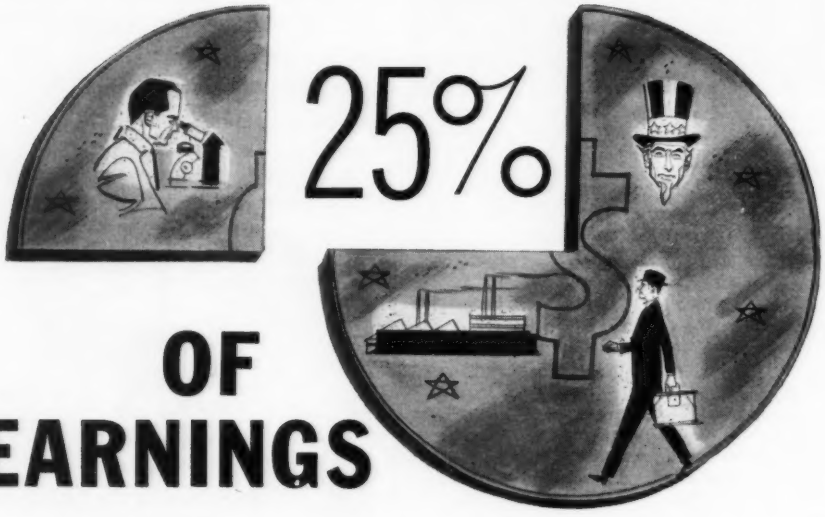

Research—the yardstick by which we prepare for the future—is of first importance at Diversey. Last year our expenditures for Research and Product Development amounted to about 25c of every dollar of pre-tax earnings.

Much of this effort is aimed at applying electronics and atomic physics to Dairy Sanitation. Advanced research like this has led to improved techniques for evaluating results of C-I-P cleaning, for instance.

In new-product development, an example of Diversey's many contributions is a new non-foaming conveyor chain lubricant—DICOLUBE SL. This lubricant provides cleaner, more smoothly operating chains and eliminates the problem of slippery, hazardous floors along conveyor lines.

By constantly searching for better products and methods—solving the small problems as well as the large ones—Diversey advances the science of dairy sanitation . . . helping to insure quality milk and improve dairy efficiency. Perhaps our experience can help you. The Diversey Corporation, 1820 Roscoe Street, Chicago 13, Illinois.

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heart, was one of the principal reasons for the move, Mr. Cherry stated.

In his annual report to shareholders, Mr. Cherry said the new subsidiary would serve expanding markets in Latin America under the name of TEISA—Cherry-Burrell, S.A., of Mexico City.

The decision to set up this subsidiary operation was based on careful investigations of the long-range industrial growth foreseen for Mexico, the increasing restrictions against importation into that country of products already made there, and the prospect of a common market embracing Mexico and her neighboring Central American republics.

Cherry-Burrell owns the new subsidiary jointly with the Mexican industrialists who owned the predecessor company, Tecnicos E Industriales, S.A. (TEISA), and who owned Cherry-Burrell's former Mexican distributor, Contratista Cherry-Burrell, S.A. The owner and manager of Contratista is sales manager of the new company. The manager of production is an experienced production man from Cherry-Burrell.

Major products of the new company will include processors, storage tanks, and fittings of the type which can not be imported into Mexico. Other products will include aluminum and steel window frames, oil drums, and other lines previously made by TEISA. In addition, the new company has been granted a license from the Heil Company of Milwaukee to make over-the-road tankers for chemical and petroleum products.

Cherry-Burrell Holds Annual Emblem Dinner

Forty-nine employees who earned their Service Emblems during 1959 were honored at the annual Cherry-Burrell Emblem dinner on January 15 at Hotel Roosevelt, Cedar Rapids, Iowa.

A total of 900 yr. of service was represented by the 49 persons from the Cedar Rapids plant, where 580 are employed. The Company's total employment, including its other plant in Little Falls, N.Y., subsidiaries in Chicago and Mexico City, and sales branches in 16 cities, is 1,650 persons.

The emblem program to honor employees with five, ten, 15, 20, 25, 30, 35, 40, and 45 yr. of service was begun in 1940 at Cherry-Burrell. Years worked at predecessor companies, including the J. G. Cherry Company, also are counted. The J. G. Cherry Co., founded in Cedar Rapids in 1880, was one of seven companies incorporated in 1928 to form the present Cherry-Burrell Corporation.

Borden's News

VIRGIL O. DREYER, a veteran Dayton, Ohio, civic and dairy industry leader, has retired

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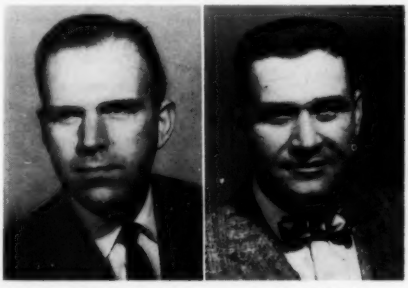
as manager of The Borden Company's Milk Division in Dayton.

Mr. Dreyer was associated with Borden's for nearly 43 yr., starting as a laboratory technician in 1917. He was graduated from Ohio State University, where he played fullback on the football team that brought OSU its first Big Ten championship. In 1956, he received the Ohio State Department of Dairy Technology's Award of Merit.

Western Condensing Expands Lactose Sales Staff

It has been announced by J. V. REGER, National Sales Manager for Lactose, Western Condensing Company, Division of Foremost Dairies, that G. D. PICKETT and J. R. BREISCH have been appointed Assistant Sales Managers for all grades of Lactose.



J. R. Breisch G. D. Pickett

Mr. Pickett, who joined the company in 1958, will be responsible for distributor sales in the western half of the country. The Chemical Department, McKesson & Robbins, Inc., is the national distributor.

Mr. Breisch will direct Lactose distributor sales in the eastern states. He started with Foremost Dairies in 1955.

Dairy Technology Societies

Atlanta—This group started off the new year with another fine program, on January 4 at the Elks Club, Decatur, Georgia. Speaker of the evening was J. T. WOODSON, JR., product sales manager, The Cherry-Burrell Company, Chicago. Their Dairy Science banquet was held Friday, January 29, at the Center for Continuing Education.

Central Michigan—January 20 was the date of the dinner meting, at High Life Inn, Saginaw, at which officers were to be installed and committees announced. Speaker of the evening was L. G. HARMON, who spoke on the problem of Antibiotics as Related to Dairy Foods.

Detroit—Annual joint meeting was held with the Allied Ice Cream Manufacturers of Metro-

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(1) Standard Methods for the Examination of Dairy Products, ed. 10, New York, American Public Health Association, 1953. (2) Committee Report, Am. J. Pub. Health 42:1131 (Sept.) 1952. (3) Microbiological Methods, report at 66th Ann. Meet. Assn. Official Agricultural Chemists, Sept. 29, 1952: J. Assn. Official Agr. Chem. 36:91 (Feb.) 1953. (4) Standard Methods for Examination of Water, Sewage and Industrial Wastes, ed. 10, New York, American Public Health Association, 1955.

Literature on B-B-L's **complete line** of culture media and apparatus for the microbiological laboratory sent on request. (13)

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politan Detroit, January 11, at the Calvert Catering Company. Speaker at this occasion was A. H. BAYER, former vice-president of the General Ice Cream Division of Sealtest Foods Company and a nationally known authority in the field of production planning and quality control.

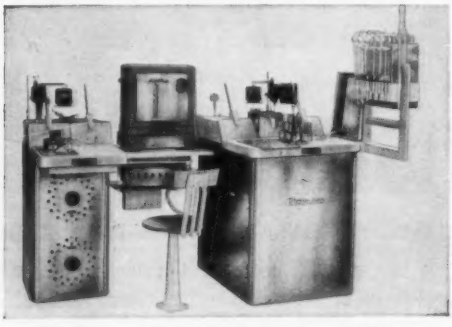
Kansas—Officers elected at the November 9th meeting, to serve during 1960, were: president—J. D. STOVAL, DeCoursey Cream Company, Wichita; first vice-president—AL EDMUNDSON, Beatrice Foods Company, Topeka; second vice-president—A. A. SAUER, State Board of Agriculture, Sabetha; sergeant-at-arms—H. V. WILKERSON, Steffen Dairy Foods, Wichita; and secretary-treasurer—T. J. CLAYDON, Dairy Department, Kansas State University, Manhattan. Committees appointed by President Stoval (chairmen are the first-named) were as follows: Membership—O. L. HONOMICHL, BRACE ROWLEY, KENNETH QUIMBY; program—PAUL CHISM, P. J. NEWMAN, C. E. CUNNINGHAM, C. L. NORTON. Educational—W. H. MARTIN, ROSS MICKELSEN, ROGER WILKOWSKE; and Reception—FLOYD KLINE, VIRGIL LAYTON, AL SLADE.

January meeting of the Society was held on the eleventh at Town and Country Lodge Restaurant, Wichita. M. E. HELD, Regional Milk and Food Consultant with the U. S. Public Health Service, Kansas City, was the speaker, his topic being Coliform Problems and Raw Milk Rating Computations. This was an opportunity for members to become better informed on a subject of much importance to the milk business.

Metropolitan—First meeting of 1960 was January 12 at Gasner's Restaurant, New York City. Principal speaker of the evening was JERRY BACK, sales engineer, Mojonnier Bros. Co., Chicago, on Materials Handling Equipment for the Dairy Industry.

Minnesota-Wisconsin—Jeantran's Supper Club, Menomonie, Wisconsin, was the locale of this joint meeting of the two societies January 18, with J. J. JEZESKI, associate professor of dairy bacteriology, department of dairy industries, University of Minnesota, as speaker. Topic: The Use of the Acid Degree Value Test in Solving Rancid Milk Flavor Problems. Recent research results in this field were mentioned, and many things of practical value and of immediate application pointed out.

North Carolina—DON R. OLSON, national sales manager of the Krim-Ko Corporation, Chicago, was the speaker at the January 13 meeting. New officers elected were: president, HENDERSON SCOTT, Melville Dairy, Burlington;



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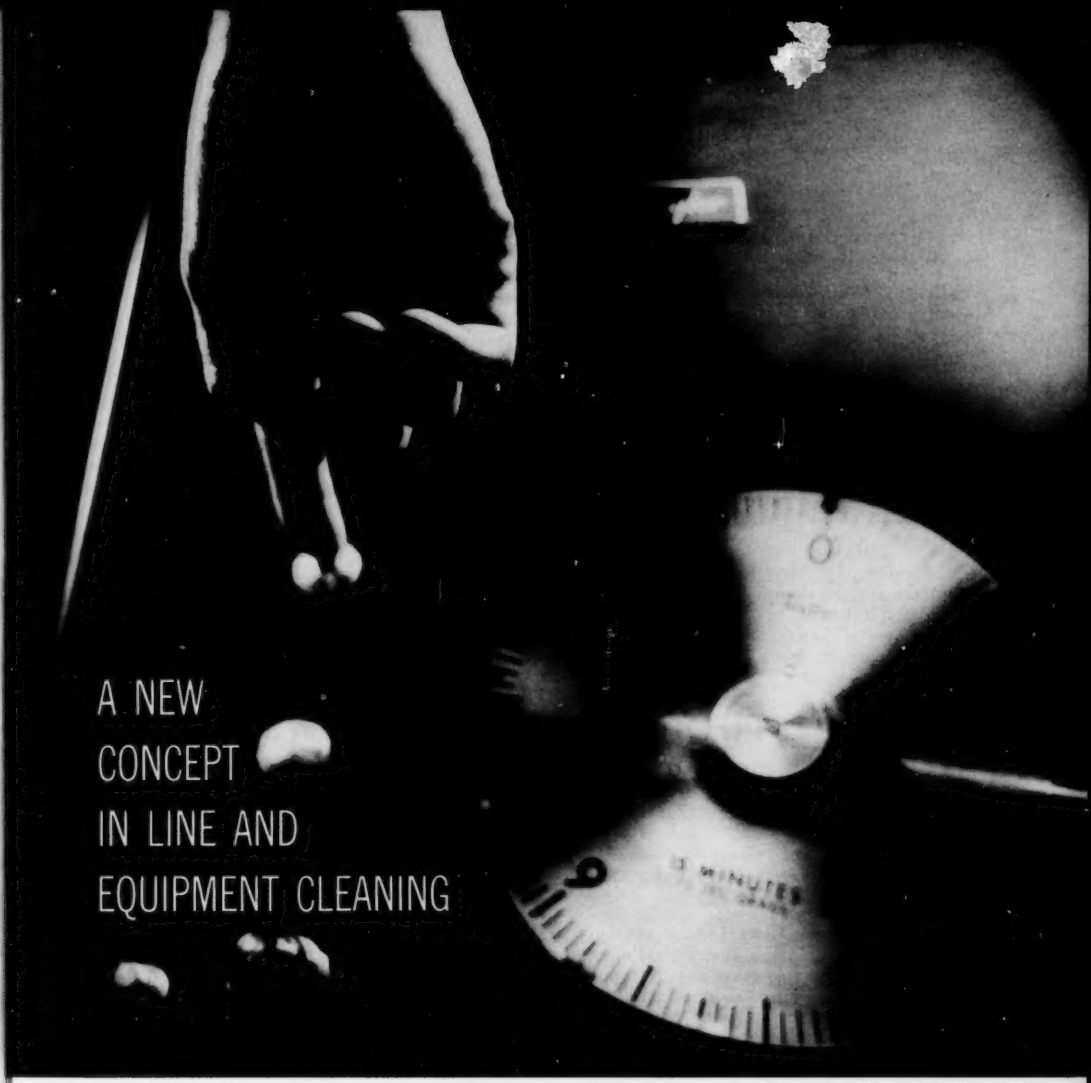

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vice-president, HERBERT COBB, Pine State Creamery, Raleigh; treasurer, VERNON WEAVER, Long Meadow Dairy, Durham, and secretary, W. M. ROBERTS, North Carolina State College.

Tri-Cities—Monthly dinner meeting was on January 12. Topic: Selling Is Our Business. Speaker: HARRY "HOP" BOWSER, Dairy Pak, Inc.

Tri-State—This group's January 27 meeting featured as speaker W. R. DILL, Assistant Dean, Graduate School of Industrial Management, Carnegie Institute of Technology, discussing Understanding Human Behavior to Better Management. Meeting-place: Mona Lisa Lounge, Whitehall.

Western Michigan—A dinner meeting was held January 18. Due to the urgency of the antibiotic and pesticide residue in milk question, DR. HARMON of Michigan State University gave a demonstration of the test for antibiotics, along with interpretation of results. The nominating committee presented a slate of nominations. DON PEOPLE, Battle Creek, will automatically become president, and the retiring president, ROBERT STEPHENSON, will serve as one of the directors. New slate: vice-president, WILLIAM RICHARDS; secretary, DON MURRAY; treasurer, WILLIAM KRUGER, sergeant-at-arms, RAY HUGHES; directors, WARD ANDERSON and FRED WEST.

Completed Theses

Ph.D. Degree

C. V. MORR—Investigation of beta-lactoglobulin and kappa-casein solutions and their interaction by means of fluorescence polarization. The Ohio State University, Columbus.

M.S. Degree

R. J. WHITE—Occurrence and control of the brown precipitate defect in making cottage cheese. Oregon State College, Corvallis.

STUDENT CHAPTER NEWS

J. E. JOHNSTON, Editor

A Section Devoted to News of Student Members

The Oregon State College Dairy Club Milk Cow Reports

At the first Dairy Club meeting of the year, it was reported that the Dairy Cattle judging team did well at the National Dairy Cattle Congress in Waterloo, Iowa, placing fifth of the 33 teams competing. The team members were HARRIS HANSEN, GENE BELT, and FRED GRIFFITH, with JOHN SEDERBURG as alternate, all under the instruction of COACH F. B. WOLBERG.

Griffith took fifth place in the over-all contest, placing second in Jerseys and tenth in Holsteins. Belt finished seventh in Guernseys and tenth in Ayrshires. The boys related to the other club members tales of a great trip, and even Coach Wolberg had to admit gaining something from the excursion.

After the contest at Waterloo, Sederburg teamed up with BOYD BISHOP and DON MESSERLE, with PHIL RUSSNOGLE as alternate and RAY KLEIWER coaching, to compete in the Dairy Cattle Judging Contest at the Pacific International Livestock Exposition. Although the boys took first in Ayrshire, Jersey, and Guernsey competition, and placed third in Holsteins and oral reasons, they fell into fourth place in the contest.

Sederburg placed fifth in Holsteins, fourth in Guernseys, and third in Ayrshires. Messerle placed second in Holsteins and first in J rseys, and Bishop placed fourth in Ayrshires and was third high man in the over-all contest. The men brought home several additions to the Dairy Club trophy case and added a few to their private collections.

The awards banquet for the Pacific International Dairy Cattle Judging Contest was held in conjunction with the Dairy Products Judging awards dinner. The products judging contest, sponsored by the Western Division of the American Dairy Science Association and the Pacific International Livestock Exposition, was conducted in the lab of the Dairy Cooperative Association, Portland, Oregon.

Judging in the products contest were JUDY BAILEY, JOE KESEY, and FRANCIS ROTHAUGE, with ED HOAK as alternate, all who received their training under the supervision of COACH ROY STEIN. In the over-all contest Miss Bailey placed fourth, while Rothauge placed sixth and Kesey tenth, to put Oregon State in second place.

The trophies were well distributed among the six teams. Oregon State placed third in cottage cheese, second in butter and cheddar cheese, and first in ice cream, while for individual placings Rothauge took third in ice cream and cottage cheese. The over-all success gave the team confidence to continue practicing for the Chicago Invitational Collegiate Dairy Products Judging Contest. They went back to criticizing cheddar cheese, butter, milk, and ice cream, but slowed down occasionally to enjoy a Dairy Club Nutty Buddy.

The Dairy Club began making Nutty Buddies, a tasty ice cream snack made of a sugar cone filled with ice cream, dipped in chocolate, and rolled in nuts, nearly 3 yr. ago. Nutty Buddy sales have been great at the ice cream counter and the dormitories have served them for Sunday dessert. Nutty Buddies and coffee, the Dairy Club's old standby, have been doing a good job of financing club activities recently. Club members have served coffee this year to a convention of Oregon sheep and wool growers and will be host to several other groups throughout the year.

Three years ago the Dairy Club bought a Guernsey heifer on a long-range club finance program. The heifer was kept at the college barns and all was well but now, 3 yr. and a bull and heifer calf later, the cow has developed difficulties. She is soon to meet the butcher, announced John Sederburg at the second club meeting.

Also at its second meeting, the club initiated nine new members, ranging from frosh to grad, bringing the club membership to near 50. Since the division of the old Dairy Department at Oregon State, and combining of Dairy Technology with Food Tech and Dairy Husbandry with Animal Husbandry, the Dairy Club has done a large part to maintain a tie between the future processors and producers of the dairy industry.

University of Tennessee Dairy Club News

The activities of the Dairy Club this year began back in September with a dinner meeting honoring the new freshmen of the Agriculture College. The high light of this program was a talk by PROFESSSOR WYLIE, head of the department for 40 yr., giving the history of the Dairy Department. Following this was a movie, The University of Tennessee.

Some other bits of pleasing information were disclosed during following meetings. One was that the 1958-59 Dairy Club Scrapbook had won third place at the last American Dairy Science Association meeting. The other being that the UT Dairy Cattle Judging Team had walked away with top honors at the Southern Intercollegiate Dairy Judging Contest, which was held at Memphis. All three of the men on the team placed in the top ten.

In October there were two noteworthy occasions. The first was Varsity Visit, at which the Dairy Club members helped serve a barbeque lunch to the FFA and FHA members from over the state. Then came the fun. A delightful wiener roast was held at Fort Dickerson Park on the chilly night of October 28. There was plenty of food and fun for everyone.

In November the club served lunch to the Knox County dairymen.

At the last meeting of the quarter, HELEN TERRY from the American Dairy Council of Knox County gave a talk on the importance of milk in the diet in comparison to other foods. The pictures for the 1959-60 Volunteer, the UT Annual, were also made at that meeting.

Plans Made for First Annual Convention

Plans are now being made for the first annual convention of the Student Branch of A.D.S.A., to be held during the Association Annual Meetings at the University of Utah in June. A committee consisting of PAUL REAVES, V.P.I., Chairman; and ART RUDNICK, JR., University of Kentucky; JAMES MICKLE, Oklahoma State University; and JESSE WILLIAMS, University of Minnesota, has been appointed to plan a program. Suggestions for the program should be sent to members of this committee. Many student chapters are now making plans to send a student representative to the convention. This is a project which requires some advance planning, since most chapters will pay the expenses of their delegate. In cases where a chapter finds it impossible to send an undergraduate delegate, either a Graduate Student or a Faculty Member should be designated as delegate to represent the chapter.

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D. G. EASTERLY, B. J. DEMOTT, AND R. G. CRAGLE
Dairy Department, University of Tennessee, Knoxville, and UT-AEC Agricultural Research Laboratory, Oak Ridge, Tennessee

SUMMARY

Milk from two Jersey cows dosed orally with strontium[89] and calcium[45] was mechanically separated into skimmilk and cream. These radioactive isotopes were removed from the skimmilk by use of an upward-flow ion exchange resin column. Dowex 50W-X12, Dowex 50W-X4, and Duolite C-20 were used. The calcium form of each resin was more effective in removal of the isotopes than was the sodium form. Between 70 and 80% of the Sr^{89} and Ca^{45} in the skimmilk was removed from the second 100 ml. passed through the column; whereas, in the tenth 100 ml., 50% of each isotope was removed, using either of the Dowex resins. The Duolite resin was not as effective when used under these conditions.

An experiment in which Sr^{89} and Ca^{45} were put directly into skimmilk and allowed to stand for 16 hr. resulted in removals which followed very closely those of the experiment with skimmilk from dosed cows. However, a higher percentage of Sr^{89} was removed than from the skimmilk of dosed cows.

Treated milk contained more calcium, had a lower pH, greater titratable acidity, and shorter rennet coagulation time than untreated milk. Resin preference of strontium over calcium was shown.

Due to the long half-life of strontium[90], and its biological similarity to calcium, it is generally regarded to be one of the principal fission products which may affect the health of humans. Since milk is the main source of calcium for most western countries, it has received attention as a source of radioactive strontium. Because of the biological similarity of Sr and Ca, both are often studied together.

Previous work at this laboratory (4) has shown that only about 0.55% of a single oral dose of Sr^{89} was secreted in the milk over a nine-day period. This figure, however, is probably somewhat dependent upon the quantity of milk secreted by the cow. A nine-day total of Sr^{89} secreted in the milk of a cow given a single oral dose closely approaches the daily total secretion from a cow given daily oral doses of the isotope. It has been shown further (4) that the ratio of Sr^{89}/Ca^{45} in milk is only about 0.15 of that found in the diet of the cow. The factors of a small amount of isotope secretion into milk from the feed, and the change in the ratio of Sr/Ca from diet to milk, show the beneficial action of the dairy cow in elimination of strontium in its conversion of roughage into human food.

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Haller and Morin (6) and Murthy and Whitney (7) found that the type and extent of modification of certain properties of milk could be controlled by using certain ion exchangers properly regenerated. Nervik *et al.* (8), using Dowex 50W in the sodium form in both the column and bulk techniques, removed nearly 90% of the tracer isotopes of calcium and strontium from processed milk to which they had added the isotopes. Investigations by Cosslett and Watts (3), reported subsequent to the completion of the work reported herein, indicated the possibility of removing, by the use of Zeocarb 225, radioactive strontium which they added to milk. Easterly *et al.* (5) have reported briefly on this subject. The object of the present investigation was to expand, and gain further information on the method.

EXPERIMENTAL PROCEDURE

Removal of strontium^{89} *and calcium*^{45} *from milk of dosed cows.* Eight consecutive daily milk samples from two Jersey cows, each given a single oral dose of 2.0 mc. of $Sr^{89}Cl_2$ and 2.5 mc. of $Ca^{45}Cl_2$, were separated mechanically by use of a DeLaval Junior Model 3300 separator and the skimmilk (noted hereafter as milk) portions passed through a cation exchange column at room temperature. The columns were 18 mm. in diameter and 61 cm. long and contained 50 g. of either Dowex 50W, 50 to 100 mesh with a cross linkage of 4%; Duolite C-20; or Dowex 50W, 50 to 100 mesh with a cross linkage of 12%. These resins are strongly acidic, cross-linked polystyrene cation exchangers. Preliminary results indicated that the conventional downflow system of column action was not feasible, because of the slow movement of the milk through the resin and the glass wool which was used to support the resin in the bottom of the column. Therefore, the upflow system was used (see Figure 1), in which milks were passed in at the bottom and out at the top of the column. Due to the turbulent action caused by the liquid entering the bottom, the resin and milk became mixed thoroughly and in such condition were not representative of a true column condition where the liquid enters the top and flows down through a settled resin bed. Flow rate was adjusted to approximately 7 ml. per minute. Slower rates resulted in channeling and faster rates resulted in the movement of the resin to the top of the column.

Both the calcium and sodium forms of each of these resins were tested for their effectiveness in the removal of Sr^{89} and Ca^{45} from milk. The resins were received in or converted into the hydrogen form and regenerated with $CaCl_2$ or NaCl of approximately 15% concentration. After about 2 liters of the solution was passed through the column, the pH of the effluent was the same as the solution entering the column. One liter of excess solution was then passed through. The columns were then washed with 200 to 300 ml. of distilled water to remove the excess $CaCl_2$ or NaCl. One liter of milk was then passed through, followed by sufficient water to remove all traces of milk. Twelve hundred to sixteen hundred milliliters of approximately 3 *N* HCl was used to elute the cations held by the resin. Regeneration was then accomplished as described above. The second, sixth, and tenth 100-ml. portions of milk coming through the

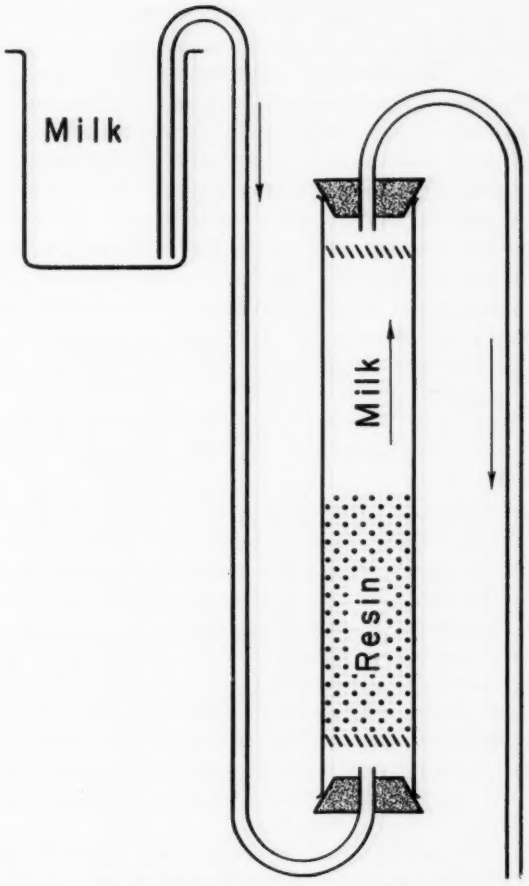

FIG. 1. Modified ion exchange column.

column were sampled for analysis. Two 25-ml. samples of milk were taken from each of the 100-ml. portions.

The samples were transferred to porcelain crucibles and dried in an oven at 90-100° C. for a minimum of 16 hr., then ashed at 600° C. overnight. Two milliliters of concentrated HCl were added to the ashed samples to bring the calcium and strontium into solution. This was transferred quantitatively to a 40-ml. centrifuge tube, precipitated as the calcium and strontium oxalates, and transferred quantitatively into a tared cup-and-tube assembly as described by Comar (2). Following centrifugation at 1,800 r.p.m. for 10 min. in a 20-in. diameter centrifuge, the supernatant was drawn off, the cup removed and dried to a constant weight at 60° C. The radioactivity of the samples was determined, using a thin mica window Geiger-Muller tube connected to a scaler unit. All

counts were for a 4-min. counting period. Differential counting of the Sr^{89} and Ca^{45} was accomplished by the use of an aluminum absorber placed between the sample and the tube. The weak Beta particles emitted from the Ca^{45} were not capable of penetration of the aluminum. A fraction of the Sr^{89} Beta particles was also absorbed (28 and 33%, respectively, for the absorbers of 54.5 and 66.6 mg/cm^2 used in these experiments). The corrected Sr^{89} count was then subtracted from the count obtained without the use of an absorber. This difference was then corrected by the method described by Comar (2) for self-absorption of the weak Beta particles emitted from the Ca^{45}. All samples were analyzed in duplicate and the average reported. The level of radioactivity in the milk from dosed cows was a maximum of approximately 3.5 μc/liter of Ca^{45} and 0.3 μc/liter of Sr^{89}. Maximum levels in the dosed milks were approximately 1.0 μc/liter of Ca^{45} and 0.6 μc/liter of Sr^{89}. These levels were calculated, using a 3% counting efficiency for the machines.

In the case of the dosed milk experiment, Sr^{89} and Ca^{45} were put into fresh raw whole milk, mixed thoroughly and left undisturbed for 16 hr. at 4° C. The whole milk was then thoroughly mixed again, heated in a water bath to 30° C., and mechanically separated. After bringing the milk to room temperature, it was passed through the column and analyzed for Sr^{89} and Ca^{45}.

Since the milk and resins were mixed thoroughly in the column, trials were conducted using the same amount of resin distributed in four columns connected in series. This arrangement had the effect of four treatments or dilutions instead of one as in the larger column. Each contained 12.5 g. of Dowex 50W, X12, 50 to 100 mesh in the calcium form. The second, sixth, tenth, and 14th 100 ml. of milk passing through this series of columns were analyzed in the same manner as described previously. This experiment was repeated four times. Following the last trial, the four columns were disconnected and the resin was washed with 1 liter of 3 *N* HCl. Samples of the eluate from each of the four columns were assayed for Sr^{89} and Ca^{45}.

Some of the physical effects of the resin treatment were determined by treating milk, containing no added isotopes, in a column containing 50 g. of Dowex 50W-X12 in the calcium form. The pH values were determined with a Beckman pH meter. The curd tensions were determined by use of a Cherry Burrell curd tension meter (1). The curd coagulation time was taken as the first sign of coagulation on those samples used for the curd tension determinations. The titratable acidity was determined by titrating 18-g. samples to the phenolphthalein end-point, using 0.1 *N* NaOH.

To determine whether the washing of the resin following regeneration had an effect upon Sr^{89} and Ca^{45} removal, columns containing 50 g. of Dowex 50W, X12 in the calcium form were flushed with 1, 2, 3, or 4 liters of distilled water following conditioning of the resin with $CaCl_2$. Milk, dosed with Sr^{89} and Ca^{45} 16 hr. previously, was then passed through the column. The second, sixth, tenth, and 14th 100-ml. portions coming through were sampled and analyzed for Sr^{89} and Ca^{45}.

RESULTS AND DISCUSSION

Values for the percentage removal of Sr^{89} and Ca^{45} from milk of dosed cows as well as from dosed milk are shown in Table 1. The removal of Sr^{89} from dosed milk was significantly greater ($P < 0.01$) than the removal from milk of dosed cows, but no difference was noted between the two milks for removal of Ca^{45}. This may have been due to the incorporation of Sr^{89} into the protein molecule in milk from dosed cows; whereas, in dosed milk, this would not likely be the case. The resins did not act the same for removal of Sr^{89} and Ca^{45} from milk of dosed cows ($P < 0.05$ and < 0.01, respectively). However, no differences were noted between resins on removal of either isotope from dosed milk. The calcium form of the resins removed a significantly greater amount of Sr^{89} than did the sodium form, with a $P < 0.01$ for milk from dosed cows and $P < 0.05$ for dosed milk. Data from the literature indicate that divalent ions are more strongly bound to a resin than monovalent ions, which would suggest a greater removal of Sr^{89} from the milk by use of resin in the Na form than in the Ca form. Data collected in the present investigations suggest the following mechanisms:

$$Sr^{89} \text{ milk} + \text{Ca resin} \rightleftharpoons \text{Ca milk} + Sr^{89} \text{ resin}$$
$$Sr^{89} \text{ milk} + \text{Na resin} \rightleftharpoons \text{Na milk} + Sr^{89} \text{ resin}$$

The calcium in the milk in the first reaction does not ionize to as great an extent as does the sodium which is in the milk in the second reaction. Because of the higher ionization constant of sodium in milk than of calcium in milk, the second reaction does not proceed as far to the right as the first.

The calcium form of the resin was more effective than the sodium form on removal of Ca^{45} from milk of dosed cows ($P < 0.05$), but no difference was noted in removal from dosed milk. This indicates that Ca^{45} from dosed cows is not as completely ionized as Ca^{45} added to milk directly. Portions passed through the column were different on Sr^{89} removal ($P < 0.01$) from both milks and on Ca^{45} removal from dosed milk. The portions were different at the $P < 0.05$ level for Ca^{45} removal from milk of dosed cows.

All three resins in the calcium form showed a slightly higher removal of Sr^{89} than of Ca^{45} for all samples tested, although the degree varied with resins and the portion passed through the columns. The removal of Sr^{89} by either of the Dowex resins decreased at a faster rate, as measured by the amount of milk passed through the column, when milk from dosed cows was used, as compared to milk which was dosed 16 hr. before exchange treatment.

Differences were noted between days of the eight-day trial period for the removal of Ca^{45} ($P < 0.01$), but not for Sr^{89} removal. Differences were noted between trials for removal of Ca^{45} ($P < 0.01$), but not of Sr^{89} from dosed milk.

The data on removal of Sr^{89} and Ca^{45} from milk by use of a series of four ion exchange columns in the calcium form are shown in Table 2. This experiment was conducted to compare the action of four columns in a series, each containing 12.5 g. of resin, to one column containing 50 g. of resin (Table 1). No differences were noted in the percentage removal of Sr^{89} and Ca^{45} between the two methods.

TABLE 1

Per cent removal of Sr^{89} and Ca^{45} from milk by exchange resin columns
(Average of eight trials)

Source of sample	100-Ml. portion of elutriated samples	Ca form of resin						Na form of resin					
		Dowex 50W-X4		Dowex 50W-X12		Duolite C-20		Dowex 50W-X4		Dowex 50W-X12		Duolite C-20	
		Sr	Ca	Sr	Ca	Sr	Ca	Sr	Ca	Sr	Ca	Sr	Ca
Dosed cows	2nd	76	75	76	74	70	64	32	49	64	68	4	39
	6th	59	50	58	56	55	48	8	31	34	51	2	33
	10th	44	39	48	46	46	41	0	18	11	31	2	26
Dosed milk	2nd	79	75	77	76	69	64	44	53	67	72	17	37
	6th	63	50	65	59	55	50	23	31	45	51	6	23
	10th	55	42	58	47	44	41	8	22	24	30	4	17

TABLE 2

Per cent removal of Sr^{89} and Ca^{45} from milk by use of a series of four exchange resin columns

Trial	100-Ml. portion of elutriated sample	Per cent Sr^{89} removal	Per cent Ca^{45} removal
1	2nd	74	75
	6th	67	63
	10th	62	51
	14th	45	38
2	2nd	77	72
	6th	52	59
	10th	57	50
	14th	55	42
3	2nd	71	73
	6th	67	52
	10th	46	30
	14th	40	31
4	2nd	82	74
	6th	73	51
	10th	51	36
	14th	36	31
Average	2nd	76	73
	6th	65	56
	10th	54	42
	14th	44	36

Following the last trial, each of the four columns of resin was washed with 1 liter of 3 *N* HCl. Forty-milliliter samples of the eluate from each of the four columns showed decreasing concentrations of both Ca^{45} and Sr^{89} in the resin from the first column to the last (Table 3). The Sr^{89}/Ca^{45} ratio decreased from the first to the last column.

Milk, without the addition of isotopes, was passed through an exchange column containing 50 g. of Dowex 50W-X12 resin. Data on eight replications for titratable acidity, curd tension, pH, and rennet coagulation time are shown in Table 4. The first 100 ml. of milk passed through the column had a lower pH, a higher titratable acidity, higher curd tension, and a lower rennet coagulation time when compared to the standard. Under conditions which removed the highest percentage of Sr^{89} and Ca^{45}, these effects were more pronounced. Therefore, as more milk was passed through the column these properties approached those of normal milk, with the exception of curd tension, which continued to increase. The shorter rennet coagulation time is partially an indication of a

TABLE 3

Radioactivity retained by resin after passage of milk containing Sr^{89} and Ca^{45}

Column	Sr^{89} counts/min/ 40 ml. eluate	Ca^{45} counts/min/ 40 ml. eluate	Sr^{89}/Ca^{45}
1	82	451	0.18
2	66	427	0.15
3	39	308	0.13
4	18	180	0.10

TABLE 4

Coagulation time, curd tension, pH, and titratable acidity of milk passed through an ion exchange column containing Dowex 50W-X12 in the calcium form

(Average of eight trials)

100-Ml. portion of elutriated sample	Coagulation time	Curd tension	pH	Acidity
	(sec.)	*(g.)*		*(%)*
Control	491 ± 23	61 ± 4	6.71 ± 0.02	0.162 ± 0.003
1	27 ± 6	82 ± 13	6.11 ± 0.07	
2			6.05 ± 0.04	0.193 ± 0.005
3	38 ± 6	122 ± 11	6.12 ± 0.04	
4			6.17 ± 0.05	0.199 ± 0.003
5	59 ± 16	128 ± 19	6.23 ± 0.04	
6			6.27 ± 0.06	0.196 ± 0.003
7	73 ± 8	142 ± 6	6.29 ± 0.05	
8			6.34 ± 0.04	0.193 ± 0.003
9	92 ± 9	151 ± 20	6.38 ± 0.05	
10			6.41 ± 0.04	0.189 ± 0.003
11	113 ± 4	131 ± 9	6.43 ± 0.04	

TABLE 5

Effect of washing of resin upon removal of Sr^{89} and Ca^{45} from dosed milk

(Average of four trials)

Liters of wash water	100th Ml. sampled	% Ca in milk	% Ca[a] removed	% Sr[a] removed	Sr[a]/Ca[a] in milk
	Not passed through column	0.138			0.637
1	2nd	0.192	80.3	84.5	0.583
	6th	0.159	77.0	76.0	0.641
	10th	0.166	61.8	68.8	0.537
	14th	0.160	51.5	54.0	0.622
	Av.	0.169	67.7	70.8	0.596
2	2nd	0.185	82.3	82.8	0.649
	6th	0.165	76.0	78.0	0.609
	10th	0.166	64.3	68.5	0.616
	14th	0.163	52.5	52.5	0.649
	Av.	0.170	68.8	70.5	0.631
3	2nd	0.180	83.5	84.3	0.614
	6th	0.165	75.8	75.5	0.663
	10th	0.164	67.3	68.0	0.631
	14th	0.160	54.8	54.3	0.653
	Av.	0.167	70.4	70.5	0.640
4	2nd	0.176	87.0	86.5	0.698
	6th	0.172	77.3	79.8	0.588
	10th	0.167	66.3	71.3	0.541
	14th	0.163	48.3	60.0	0.602
	Av.	0.170	69.7	74.4	0.607

[a] Radioactive.

higher calcium content in treated milk. The lower pH is explained by the fact that as calcium concentration increases it tends to form calcium hydrogen phosphate, releasing hydrogen ions from calcium dihydrogen phosphate. Milk so treated (without added radioactivity) was tasted and the first few samples coming through the column were found to be slightly bitter.

Ion exchange resins as used in these trials increased the calcium content of the milk passed through the column. As shown in Table 5, the average calcium content of the milk used in these trials was 0.138%, and the calcium content of the milk after passing through the resin column was about 0.170%. The first portion passing through the column contained more calcium than subsequent portions. The quantity of water with which the resin was washed appeared to have little influence. However, the first milk passed through was somewhat higher than subsequent portions with a limited amount of washing; whereas, with more washing, the samples of milk coming through were more uniform in calcium content.

The percentage of Ca^{45} removed from the milk did not seem to be influenced by the quantity of wash water used, but in Sr^{89} removal the column which was washed with 4 liters of water removed approximately 4% more than any of the three other columns. A slightly greater removal of Sr^{89} than Ca^{45} was obtained, indicating a preference of the resin for strontium over calcium. This is in keeping with the theory that resin preference for ions follows the lyotropic series pattern. Except for the column washed with 3 liters of water, all showed a slightly lower Sr^{89}/Ca^{45} ratio than in the milk before treatment. This further indicates a preference of the resin for strontium over calcium.

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STRONTIUM IN MILK. II. REMOVAL BY BATCH ION EXCHANGE METHODS [1]

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SUMMARY

Skimmilk containing Sr^{89} and Ca^{45} was left in contact with Dowex 50W-X12 in the calcium form for periods of 1, 10, 60, and 120 min. Apparent equilibrium was reached between the 1- and 10-min. periods. A dosed milk-resin ratio study showed that as the ratio increased, the percentage of removal of Sr^{89} and Ca^{45} decreased. The percentage removal ranged from a high of 94 and 93 for Sr^{89} and Ca^{45}, respectively, at a milk-resin ratio of 2, to a low of 21% Sr^{89} and 16% Ca^{45} at a milk-resin ratio of 200. At a milk-resin ratio of 2, 4.27% of the resin capacity was utilized and at a ratio of 200, 73.6% was utilized for exchange of Sr and Ca.

Nervik *et al.* (3) have shown that resins in the sodium form are capable of removing radioactive strontium from milk and Easterly *et al.* (1, 2) have shown that resins in the calcium form are capable of removing a large amount of the Sr^{89} contained in milk from cows fed the isotope. The latter work was done in a continuous reverse-flow column system in which the milk-resin ratio and the contact time could be determined only by derivation and not directly. The present study was undertaken to show the effects of various milk-resin ratios, the time necessary for apparent equilibrium, and the milk-resin exchange characteristics between Sr^{89} and Ca^{45}.

EXPERIMENTAL PROCEDURE

Dowex 50W-X12 in the calcium form was used in these studies. To determine the length of time necessary for milk-resin equilibrium, raw skimmilk (hereafter referred to as milk) was dosed with approximately 0.6 μc. of Sr^{89} Cl_2 and 1.0 μc. of Ca^{45} Cl_2, mixed thoroughly and left undisturbed for 16 hr. at 4° C.

Five grams of the resin, in the hydrogen form as received, was rinsed at least ten times with 100-ml. lots of $CaCl_2$ of approximately 15% concentration, followed by three washings of 100 ml. each with distilled water. The liquids were removed from the resin by decantation. Each trial was conducted with fresh resin.

After heating the milk to room temperature, 100 ml. was added to each of the four beakers, each containing 5 g. of the prepared resin. After 1 min. of mixing, Sample One was filtered to remove the resin and two 25-ml. aliquots

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taken. The same procedure was followed for Samples Two, Three, and Four after 10, 60, and 120 min. of agitation, respectively. All samples were mixed by hand for the first minute and by use of a mechanical agitator thereafter. Two 25-ml. aliquots having no resin contact were used as standards to calculate the percentage removal of Sr^{89} and Ca^{45} for each contact time. The radioactivity of the aliquots was determined as described by Easterly *et al.* (2).

To determine the time necessary for equilibrium of Sr^{89} and Ca^{45} with the Sr and Ca normally present in milk, four 100-ml. samples of raw milk at room temperature were dosed with the isotopes and mixed for 1, 10, 30, or 60 min. Each sample was then exposed to 5 g. of the resin for 60 min., filtered, and analyzed for radioactivity.

The milk-resin ratio studies were made with milk dosed with Sr^{89} and Ca^{45} 16 hr. before treatment. After heating to 25° C., six 200-ml. samples were mixed with 1, 5, 10, 20, 40, or 100 g. of resin. After mechanical agitation for 4 hr., the samples were filtered and analyzed.

RESULTS AND DISCUSSION

Data on the removal of Sr^{89} and Ca^{45} from milk as influenced by the milk-resin contact time are presented in Table 1. The average of three trials showed 62% Sr^{89} and 52% Ca^{45} removal after a contact time of 1 min.; whereas, 76% Sr^{89} and 67% Ca^{45} were removed after 120 min., the greatest difference being between the 1- and 10-min. periods ($P<0.05$).

The data for the removal of Sr^{89} and Ca^{45} as influenced by the isotope-milk contact time are presented in Table 2.

Results show a greater removal of Sr^{89} than Ca^{45}, with very little difference between that agitated for 1 min. and that agitated for 60 min. before exchange treatment. The isotopes are fully incorporated into the milk within 1 min. or

TABLE 1

Effect of resin-milk contact time on per cent removal of Sr^{89} and Ca^{45} from dosed milk

Trial	Resin-milk contact time	Sr^{89} removal	Ca^{45} removal
	(min.)	*(%)*	
1	1	66	56
	10	69	54
	60	77	70
	120	76	69
2	1	57	44
	10	71	64
	60	68	55
	120	78	70
3	1	64	55
	10	75	62
	60	77	72
	120	75	60
Av.	1	62	52
	10	72	60
	60	74	66
	120	76	67

TABLE 2

Effect of isotope-milk contact time on per cent removal of Sr^{89} and Ca^{45} from dosed milk

Trial	Isotope-milk contact time	Sr^{89} removal	Ca^{45} removal
	(min.)	*(%)*	
1	1	78	71
	10	79	70
	30	76	72
	60	72	61
2	1	83	78
	10	84	77
	30	73	71
	60	80	70
3	1	83	81
	10	73	73
	30	82	87
	60	81	78
4	1	80	73
	10	78	67
	30	83	74
	60	73	61
Av.	1	81	76
	10	79	72
	30	79	76
	60	77	68

the rate of incorporation at this stage is so slow that 60 min. is not sufficient time to show a definite trend. The latter is likely, since the average per cent removal of the isotopes was less after contact with the milk for 16 hr. (Table 1) than 60 min. (Table 2).

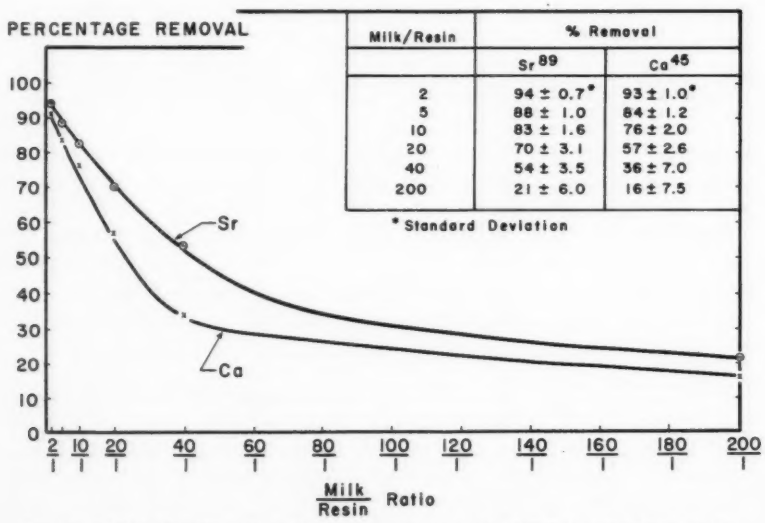

FIG. 1. Effect of milk-resin ratio on per cent removal of Sr^{89} and Ca^{45} from milk.

Figure 1 indicates a direct relationship between the percentage of Sr^{89} and Ca^{45} removed from milk and the milk-resin ratio used. The percentage removal of Sr^{89} ranged from a high of 94, where two parts of milk were used per one part of resin, to a low of 21, where 200 parts milk per one part of resin were used. Similarly, the percentage Ca^{45} removal ranged from a high of 93 to a low of 16. The total resin exchange capacity (meq/gram), as given by the manufacturer, was approximately twice the amount required for complete removal of the calcium from the milk at a milk-resin ratio of 20. At this ratio, the average percentage removal of Sr^{89} was approximately 70 and Ca^{45} was 57.

Relationships between resin weight and exchange of Sr^{89} and Ca^{45} from milk are useful for predicting removal using different ratios. With the resin in the calcium form, the removal of Ca^{45} from the milk should logically be on the basis of isotope dilution. Such is shown to be the case (Figure 2), but the removal of Sr^{89} does not follow this pattern, indicating other factors are involved.

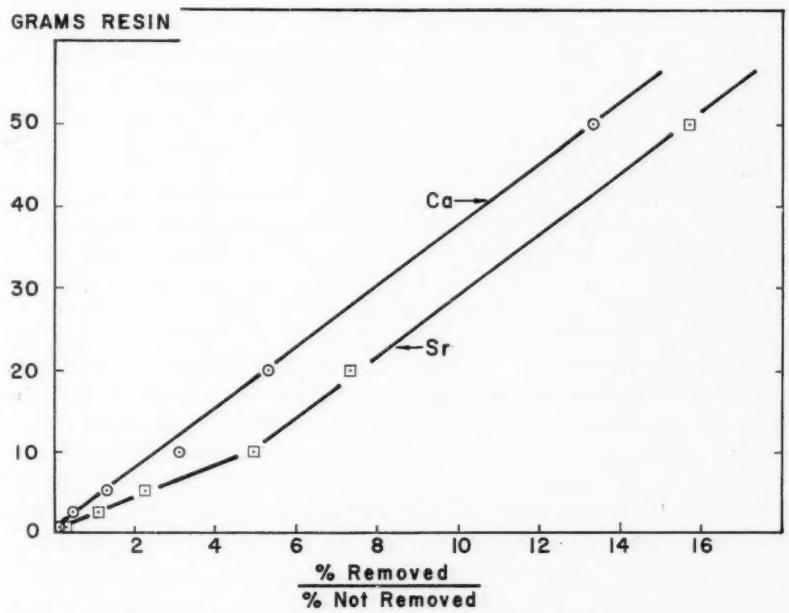

FIG. 2. Relationship between grams of resin per 100 ml. of milk and the ratio of $\frac{\%\text{ removed}}{\%\text{ not removed}}$ of Sr^{89} and Ca^{45}.

On the basis of the calcium oxalate weights, most milk samples treated with the resin in the calcium form contained more calcium than the nontreated milk. The increase was greater for the low milk-resin ratios. Using ratios of 2, 5, 10, 20, 40, and 200, the average of three trials showed increases in calcium

TABLE 3

Per cent of resin capacity exchanged by calcium and strontium using the batch and column system

System used	Milk-resin ratio or portion through column	Isotope removed	Removed	Resin capacity exchanged
			(%)	
Batch	2–1	Ca^{45}	93	4.27
Batch	5–1	Ca^{45}	84	9.6
Batch	10–1	Ca^{45}	76	17.4
Batch	20–1	Ca^{45}	56	26.2
Batch	40–1	Ca^{45}	34	31.3
Batch	200–1	Ca^{45}	16	73.6
Batch	2–1	Sr^{89}	94	..... [b]
Column	1st 100 ml.	Ca^{45}	82	3.77
Column	1st 100 ml.	Sr^{89}	81	..... [b]
Column [a]	1st 100 ml.	Sr^{89}	81	..... [b]
Column [a]	1st 100 ml.	Ca^{45}	74	3.41

[a] Milk from dosed cows. All other samples were dosed milk.
[b] Stable strontium in milk is negligible compared to calcium.

contents of: 57.3 ± 3.2, 22.1 ± 1.7, 21.9 ± 0.7, 22.0 ± 1.7, 5.4 ± 13.1, and $3.3 \pm 8.5\%$, respectively. The large standard deviation on the latter two averages reflects the fact that some milk samples lost calcium to the resin. The increases may be due to the excess calcium chloride not being washed from the recharged resin prior to milk contact. Some additional calcium also comes from the exchange of calcium on the resin for ions other than Ca in milk.

An estimation of the efficiency of the column system versus the batch system was made. Equations for the removal of Sr^{89} and Ca^{45} from dosed milk and milk of dosed cows were derived by the method of least squares from data presented earlier (2). The per cent removal of isotope, (y axis), was plotted against the quantity of milk passed through the column (x axis). From the integrated forms of these equations between the limits of 0 and 100 ml., the area under the curve was determined. A comparison of these systems is presented in Table 3. The data on the percentage removal of the isotopes in the column system are derived values and correspond to a milk-resin ratio of 2. On this basis, it is estimated that the column was 86% as efficient as the batch system for removal of Sr^{89}. The corresponding value for Ca^{45} was 88%. The lower efficiency for the column might be attributed to the milk-ion and resin-ion complex, being further from equilibrium than in the case of the batch system.

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A METHOD FOR THE ELIMINATION OF ASHING IN STRONTIUM^{90} DETERMINATION OF MILK

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SUMMARY

A method is described which eliminates the necessity for ashing in the determination of strontium^{90} in milk. After the addition of a known amount of strontium carrier, the milk is treated with an equal volume of 24% trichloroacetic acid. The solution is filtered and the alkaline earths precipitated from the filtrate by adjusting the pH to 8.5–9.0 with NaOH in the presence of Na_2CO_3. The alkaline earth precipitates are separated and analyzed as described previously for milk ash (8). The results of strontium^{90} analysis on alkaline earth precipitates are not significantly different from the results obtained by analysis of milk ash.

Several of the methods (1, 3, 6, 8) currently used for the determination of strontium^{90} in milk require ashing of large quantities of sample. These procedures are invariably time-consuming and often require special apparatus, including rapid-ashing devices (1, 7) and large muffle furnaces. Therefore, elimination of ashing would greatly simplify the routine determination of strontium^{90} and other nonvolatile radionuclides.

Although nonashing procedures (4, 9) have been developed to determine the cationic composition of milk, they require large dilutions of the original sample, which make them impractical for processing the quantities required in radionuclide analyses. On the other hand, the method of preparing samples in the determination of citric acid (2, 10), using a trichloroacetic acid (TCA) precipitation of milk proteins, seemed promising. Accordingly, the possibility of using this technique in connection with the determination of strontium^{90} was explored, resulting in a simplified method for preparing samples for analysis. This method yields results comparable to those requiring ashing, but providing considerable savings in time and equipment.

METHODS

One liter of milk at 20° C. is placed in a 2-liter flask, and 1 ml. of 1 *M* $Sr(NO_3)_2$ is added and mixed well. This solution is treated with an equal volume (1 liter) of 24% TCA while stirring, and allowed to stand for 15 min. The precipitated proteins are separated by filtration through a Whatman No. 12 filter paper and the filtrate is collected in a 2-liter beaker. Four or five drops of a mixed indicator solution (0.1 g. of thymol blue + 0.1 g. of phenolphthalein in 100 ml. of 50% ethyl alcohol) are added and the alkaline earths precipitated by adjusting the filtrate to pH 8.5–9.0 with 6 *N* NaOH, after which 50 ml. of 3 *N* Na_2CO_3 is added. The solution is mixed well and allowed to stand for at least 4 hr. to permit the alkaline earth precipitates to settle. The clear supernatant is siphoned off with suction and discarded. The remaining solution is centrifuged

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in 250-ml. bottles at 1,800 r.p.m. for 15 min. The resulting supernatant is discarded and the precipitate washed once with 200 ml. distilled water, recentrifuged, and the wash water discarded. The precipitate is quantitatively transferred to a 250-ml. beaker with dilute HNO_3.

Determination of strontium^{90}. The method used for the determination of strontium^{90} is the same as that employed for milk ash, described by Murthy *et al.* (8).

RESULTS AND DISCUSSION

Composition of milk and TCA filtrate. To determine the feasibility of the TCA fractionation procedure, it was necessary to establish that the cationic composition of the original milk and the TCA filtrate were the same.

Sufficient milk was obtained to make a comparison on a common sample. Calcium and potassium were selected as representative cations, in view of their similarity to strontium and cesium. The proteins were precipitated from 100-ml. aliquots in the manner described under Methods, with appropriate adjustments in volume. The protein fraction was washed three times by dispersion in 100-ml. portions of 12% TCA. Samples of the original milk, the washed protein, and the TCA filtrate were ashed overnight in a muffle furnace at 550° C. The ashed samples were dissolved in dilute HCl and the calcium and potassium content of each was determined, using a Beckman Model DU flame spectrophotometer. The concentration of calcium and potassium in the TCA filtrate was corrected for dilution and the results are summarized in Table 1. It is seen that the

TABLE 1

Calcium and potassium content of milk, TCA filtrate, and milk proteins

		Calcium	Potassium			Calcium	Potassium
		—(gm./liter)—				*—(gm./liter)—*	
Whole milk		1.379	1.439	Milk proteins	1	0.0054	N.D.
TCA filtrate	1	1.384	1.433		2	0.0054	N.D.
	2	1.399	1.478		3	0.0095	N.D.
	3	1.370	1.438		4	0.0071	N.D.
	4	1.378	1.463	Average		0.0068	N.D.
Average		1.383	1.453				

N.D. = not detectable.

calcium and potassium content of the original sample and of the TCA filtrate are essentially identical within the limits of error of the methods. The concentration of calcium in the protein fraction confirms that the losses in the precipitate are negligible and may be ignored. These findings indicate that the TCA fractionation is, therefore, satisfactory for the separation of proteins from the cations in milk.

Recovery of strontium in the alkaline earth precipitate. The alkaline earths were precipitated according to the technique described in Methods, from several 500-ml. portions of milk to which a known amount of strontium^{85} had been added. The precipitates were separated, dissolved in 100 ml. of dilute HCl, and their gamma activity determined in a scintillation spectrometer. The ob-

TABLE 2
Recovery of Sr^{85} added to milk

Alkaline earth fraction				Alkaline earth fraction			
Sample	Added	Observed	Recovery	Sample	Added	Observed	Recovery
	—(counts/min)—		(%)		—(counts/min)—		(%)
1	3,570	3,550	99.7	4	3,570	3,620	101.3
2	3,570	3,450	96.8	5	3,570	3,630	101.6
3	3,570	3,620	101.3	6	3,570	3,570	99.9
							Av. 100.1 ± 1.8

served activities were corrected for dilution and these results are summarized in Table 2. Inspection of these data reveals that strontium^{85} was quantitatively recovered from the milk and indicates that the method is satisfactory for the isolation of available strontium.

Comparison of the precipitation method with the ashing technique. To determine whether the results obtained with the precipitation method are reproducible and comparable to those obtained from the ashing procedure, the strontium^{90} content of 22 different milk samples was determined by both methods and the results are presented in Table 3. In calculating the concentration of strontium^{90} in the milk samples, the observed values were corrected for losses encountered in the isolation of $SrCO_3$. This correction factor was the ratio of the strontium in the isolated sample, as determined by flame photometry, to the amount of strontium carrier added before processing. The feasibility of basing the correction on the chemical recovery of the added carrier was demonstrated through

TABLE 3
Sr^{90} content of milk by precipitation and ash methods

	Sr^{90} content			Sr^{90} content	
Sample	Ash method	Precipitation method	Sample	Ash method	Precipitation method
	—(μμc/liter)—			—(μμc/liter)—	
1	9.0 ± 0.9	10.3 ± 0.7	12	20.8 ± 1.1	20.0 ± 0.8
2	14.0 ± 1.0	12.7 ± 1.1	13	7.3 ± 0.5	9.4 ± 0.9
3	10.2 ± 0.7	9.1 ± 0.9	14	8.9 ± 0.7	11.0 ± 0.8
4	8.6 ± 1.0	8.3 ± 0.9	15	12.3 ± 0.9	14.8 ± 0.8
5	12.3 ± 1.1	13.9 ± 1.0	16	6.3 ± 0.6	7.6 ± 0.9
6	6.5 ± 0.7	6.9 ± 0.8	17	5.5 ± 0.7	5.9 ± 0.9
7	7.6 ± 0.7	8.8 ± 0.7	18	3.1 ± 0 3	4.5 ± 0.5
8	16.5 ± 0.8	15.5 ± 0.9	19	6.0 ± 0.7	4.0 ± 0.8
9	19.6 ± 1.1	20.8 ± 1.1	20	7.5 ± 0.7	8.3 ± 0.7
10	37.3 ± 3.8	34.3 ± 4.0	21	2.3 ± 0.6	3.0 ± 0.6
11	7.5 ± 0.7	8.3 ± 0.6	22	3.2 ± 0.5	3.0 ± 0.6

Analysis of variance

Source	Degrees of freedom	Sum of squares	Mean square	Variance ratio	Significance
Method	1	1.491	1.491	0.06	N.S.
Milk	21	1,769.933	84.282	3.14	V.S.
Error	21	564.004	26.850		
Total	43	2,335.428			

N.S. = not significant; V.S. = very significant.

TABLE 4
Strontium content of milk

Source of sample	Strontium concentration	Source of sample	Strontium concentration
	(mg/liter)		*(mg/liter)*
Austin, Texas	0.15	New York, New York	0.10
Fargo, North Dakota	0.07	Provo, Utah	0.55
Farmington, New Mexico	0.33	Salt Lake City, Utah	0.39
Los Angeles, California	0.15	Spokane, Washington	0.29
		St. Louis, Missouri	0.16

neutron inactivation analysis (5) of selected milk samples for strontium. The results are presented in Table 4. It is seen that the strontium present in milk is less than 1% of the added strontium carrier (87.6 mg.). Since the errors in the flame photometric determination of strontium are approximately $\pm$ 2%, it is evident that the contribution of strontium in the milk may be neglected in calculating the total recovery of the element.

Statistical analysis of the data in Table 3 demonstrates that the strontium^{90} content of milk samples obtained by analysis of either alkaline earth precipitates or milk ash is not significantly different. Therefore, it is concluded that the plan of analysis described here may be employed for the determination of strontium^{90} in milk.

ACKNOWLEDGMENTS

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SOLUBILITY OF TRICALCIUM CITRATE IN SOLUTIONS OF VARIABLE IONIC STRENGTH AND IN MILK ULTRAFILTRATES [1]

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SUMMARY

Solubility of tricalcium citrate has been determined at 21 and 95° C. by dissolution in water and by precipitation from supersaturated solutions containing various proportions of calcium to citrate and at pH 4.4 to 8.8. Solubility product in solutions at equilibrium varied with ionic strength, according to the relation $pk_s = 17.63 - 10.84\sqrt{\mu}$, but was unaffected by variations in pH and temperature and by the presence of magnesium or phosphate ions. Milk ultrafiltrates were shown to be saturated with tricalcium citrate, i.e., the calculated pk_s values agreed with the solubility product in all but two of the 15 samples tested, and composition of two ultrafiltrates was unaffected by agitation with crystals of tricalcium citrate.

Few data are in the literature on the solubility of calcium citrate. It is generally believed that precipitation will not occur from moderately supersaturated solutions of calcium citrate at room temperatures and that it is necessary to heat the supersaturated solutions to about 95° C. to initiate precipitation (2, 22, 24). Crystals of tricalcium citrate obtained from concentrated solutions at 30° C. were 6-hydrate, whereas those obtained at 95° C. were 4-hydrate (2). The 6-hydrate was unstable and gradually converted to the 4-hydrate in air at room temperature (2). Solubility of the two hydrates at various temperatures, determined by agitating the crystals in water, has been reported (on anhydrous basis) as:

Partheil and Hubner (20)

4-hydrate	18° C.	0.742 g/liter
4-hydrate	25° C.	0.838 g/liter

Chatterjee and Dhar (2)

6-hydrate	30° C.	2.01 g/liter
4-hydrate	30° C.	2.20 g/liter
4-hydrate	95° C.	1.83 g/liter

Solubility of calcium citrate has not been determined in milk ultrafiltrates or in solutions of comparable ionic strength and pH. Crystals of tricalcium citrate have been observed in evaporated milk (5), sweetened condensed milk (13), and in spray-dried skimmilk powder (7). The present paper reports results of solubility measurements at 21 and 95° C. in solutions of various ionic strengths and pH values, and illustrates the effect of magnesium and phosphate on the solubility. Concentration products of tricalcium citrate in milk ultrafiltrates have been calculated from published data and from data obtained in this laboratory.

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MATERIALS AND METHODS

Two methods were used to determine the solubility of calcium citrate:

(1) Solutions containing various concentrations (10-78 m*M*/liter) of citric acid and calcium chloride were adjusted to pH between 4.3 and 9.0 by adding sodium hydroxide, and were seeded with crystals of tricalcium citrate and then agitated continually during crystallization. Ionic strengths of supernatants were calculated from the initial chloride and sodium concentrations and the level of residual calcium and citrate.

(2) To obtain the solubility of the salt at low ionic strength, crystals of tricalcium citrate were agitated with distilled water.

In both methods, crystallization or dissolution at 21° C. was considered complete when the composition of supernatant remained constant for at least two days.[2] At 95° C., crystallization and dissolution of calcium citrate are rapid (2) and were assumed to be complete after 1 hr. with occasional agitation.

To study the effect of magnesium on the solubility of calcium citrate, 3 m*M* of magnesium chloride per liter was added to the supersaturated calcium chloride–trisodium citrate solutions and the pH adjusted to approximately 8.8. To study the effect of phosphate, additions of an equimolar mixture of mono- and dipotassium phosphate (1 *M*) were made to solutions already equilibrated at pH 6.8–7.0 with tricalcium citrate crystals. The solutions containing phosphate were agitated for a further three days to insure equilibrium under the new condition. The concentrations of phosphate chosen (3–10 m*M*/liter) did not induce precipitation of calcium phosphate in the calcium citrate solutions.

For analysis, supernatants were separated from crystals either by centrifugation or by filtration. Calcium was determined by a turbidimetric method (14), citrate by colorimetry (15), calcium ion with the ammonium purpurate method (27), pH with the glass electrode, magnesium by a method based on the turbidity formed by potassium erucicate (16), and phosphate by the ammonium phosphomolybdate–stannous chloride method of Polley (21).

CALCULATIONS

Solubility of tricalcium citrate was calculated as the product $k_s = (Ca^{++})^3 \times (Cit^{\equiv})^2$. Concentations of Ca^{++} and $Cit^{\equiv}$ ions are lower than the total concentrations of calcium and citrate in the supernatants because of formation of $[CaCit]^-$ complex (8), but could be calculated (when no other complexing ions are present) from the calcium and citrate concentrations, using known dissociation constants of citric acid and calcium citrate. Two methods of calculation were used, depending on whether the pH was above or below the value for complete dissociation of citric acid (*ca.* pH 7). At pH above 7, the following known equations (8) were used:

$$Ca^{++} = \frac{-(K_4 + Cit - Ca) + \sqrt{(K_4 + Cit - Ca)^2 + 4\,K_4Ca}}{2} \qquad (1)$$

since $[CaCit]^- = Ca - Ca^{++}$, and $Cit^{\equiv} = Cit - [CaCit]^-$

then $Cit^{\equiv} = Cit - Ca + Ca^{++}$ (2)

[2] In this work, a 1% v/v addition of chloroform was added, to prevent bacterial destruction of citrate.

where Ca and Cit represent the total concentrations of calcium and citrate in moles per liter, K_4 is the dissociation constant of $[CaCit]^-$, and brackets [] indicate a complex ion.

At pH's below 7, several types of acid and salt dissociations occur (4), the most important quantitatively being:

$$\text{i)} \quad \frac{[H_2Cit]^- \times H^+}{[H_3Cit]^\circ} = K_1 \quad \frac{[HCit]^= \times H^+}{[H_2Cit]^-} = K_2 \quad \frac{Cit^\equiv \times H^+}{[HCit]^=} = K_3$$

and:

$$\text{ii)} \quad \frac{Ca^{++} \times Cit^\equiv}{[CaCit]^-} = K_4 \qquad \frac{[CaCit]^- \times H^+}{[CaHCit]^\circ} = K_5$$

These were considered in the following calculations of Ca^{++} and $Cit^\equiv$ concentrations in solutions at pH values of less than seven. The concentration of $Cit^\equiv$ was calculated from the three dissociation constants of citric acid (11, 17):

$$Cit^\equiv = \frac{Cit_N K_1K_2K_3}{H^{+3} + H^{+2}K_1 + H^+K_1K_2 + K_1K_2K_3} \tag{3}$$

The net citrate was calculated as:

$$Cit_N = Cit - [CaCit]^- - [CaHCit]^\circ$$

and by substituting (ii),

$$Cit_N = Cit - \frac{Ca^{++} \times Cit^\equiv}{K_4} - \frac{Ca^{++} \times Cit^\equiv \times H^+}{K_4K_5} \tag{4}$$

Concentration of calcium ion can be expressed from total calcium as:

$$Ca = Ca^{++} + [CaCit]^- + [CaHCit]^\circ$$

and, by substitution of (ii)

$$Ca^{++} = \frac{Ca\, K_4K_5}{Cit^\equiv K_5 + Cit^\equiv H^+ + K_4K_5} \tag{5}$$

Substituting Equation (4) and then (5) in Equation (3), and solving for trivalent citrate ions, we obtain the following equation:

$$Cit^\equiv = \frac{-b + \sqrt{b^2 + 4ZK_4K_5Cit\ (K_5Y + H^+Y)}}{2\ (K_5Y + H^+Y)} \tag{6}$$

Where $b = K_4K_5Y + ZK_5Ca + ZH^+Ca - ZK_5Cit - ZH^+Cit,$

$$Y = H^{+3} + H^{+2}K_1 + H^+K_1K_2 + K_1K_2K_3,$$

$$Z = K_1K_2K_3$$

The citric acid dissociation constants used where those reported in the literature for ionic strengths and temperatures close to those existing in the supernatants studied: K_1: 1.19×10^{-3}; K_2: 4.30×10^{-5}; K_3: 1.73×10^{-6} for μ of 0.1

and a temperature of 18° C. (1), and K_5: 2×10^{-5} for μ of 0.08 and a temperature of 37° C. (19). K_5 values were not found for ionic strengths other than 0.08 and, therefore, the study of solubility at low pH was done with solutions close to that ionic strength only. K_4 was used with supernatants of various ionic strengths and its value was, therefore, calculated for each supernatant from the relationship $pK_4 = 4.64 - 3.64 \sqrt{\mu}$, derived from pk_4 values determined by various techniques (4, 8, 9, 18, 19, 25) at temperatures around 20° C. and ionic strengths between 0 and 0.16.

Magnesium, when present, competes with calcium for the binding of citrate but, because the citrates of magnesium and calcium are about equally dissociated (8, 19), it can be shown that substituting the sum of Ca + Mg for Ca in Equations (1), (6), and (5), will give the sum of $Ca^{++} + Mg^{++}$ in Equations (1) and (5). Since Ca^{++}/Mg^{++} will be approximately equal to Ca/Mg, the Ca^{++} and Mg^{++} concentrations can be calculated, once the sum is known.

It can be estimated (6) that, usually, about 1 m*M* of calcium or magnesium per liter is bound by phosphate in solutions comparable to milk serum. Although small, this binding can not be neglected, because it might reduce Ca^{++} concentration significantly. The amount of calcium (or Ca + Mg) bound was, therefore, calculated, assuming that Ca^{++} (or Mg^{++}) and $HPO_4^{=}$ associate in the ratio of 1 : 1 to form $CaHPO_4$ and that the concentration of other forms of undissociated calcium phosphate was negligible.

Since
$$\frac{Ca^{++} \times HPO_4^{=}}{CaHPO_4} = K_p$$

$$\frac{HPO_4^{=} \times H^{+}}{H_2PO_4^{-}} = K_A$$

and
$$P = H_2PO_4^{-} + HPO_4^{=} \text{ (between pH 5 and 8)}$$

therefore, by substitution:

$$CaHPO_4 = \frac{Ca^{++} \times P \times K_A}{(K_A + H^{+}) \times K_p} \qquad (7)$$

where P is the total phosphate concentration, K_A is the second acid dissociation constant of phosphoric acid, i.e., 1.68×10^{-7} (10), and K_p is the dissociation constant of $CaHPO_4$, i.e., 1.5×10^{-2} (6).

In solutions containing calcium, citrate, and phosphate, the calculation of Ca^{++} and $Cit^{\equiv}$ concentrations was made as follows: A first approximation of Ca^{++} was made, using Equations (6) and (5); concentration of $CaHPO_4$ was then calculated from Equation (7) (two approximations were made) and then subtracted from the concentration of total calcium; finally, the net calcium concentration was used in Equations (6) and (5) to calculate the second approximation of Ca^{++} and $Cit^{\equiv}$ concentrations. A greater number of approximations did not change the results significantly. Where magnesium also was present in the solution, the sum of concentrations of Ca + Mg was used in the calculations instead of Ca alone.

RESULTS AND DISCUSSION

Calcium citrate solutions. Crystals started to appear in supersaturated solutions of calcium citrate after about two days at 21° C. and continued to form for about 12 days. Seeding and agitation reduced crystallization time to three or four days over-all. However, to obtain rapid crystal growth in solutions that were only slightly supersaturated, it was found that at least 25 mg. of calcium citrate (anhydrous basis) per 100 ml. of solution had to be introduced as seed crystals. At 95° C., crystals appeared rapidly and crystallization appeared complete in less than 1 hr. The crystals obtained at both temperatures were examined microscopically and were found to have the appearance of clusters of needles. The molar calcium/citrate ratio 1.497 ± .014 was that of tricalcium citrate.

The instability of water of crystallization at normal experimental temperatures prevented its accurate determination. However, no difference was found in the amount of water of crystallization, nor in the rate of dehydration, between crystals obtained at 21 and 95° C. Crystals kept for ten days in open air and then ten days over P_2O_5 at 25° C. lost over 60% of their water of crystallization. Complete dehydration was accomplished by heating samples overnight in a vacuum oven at 100° C.; further heating at 130° C. did not change the weight. Other workers (2) have found up to 16 molecules of water of crystallization in crystals obtained at room temperature from highly supersaturated solutions, and four waters in crystals obtained at 95° C.

The relation between calcium and citrate concentrations in the supernatants at equilibrium at pH 7 or above is shown in Figure 1. The curve passed through a minimum at a point corresponding to 6 m*M* of calcium and 3 m*M* of citrate per liter. The increased concentration of calcium at higher citrate concentrations was due partly to binding of calcium by citrate, and partly to increased solubility owing to increased ionic strength.

Analysis of supernatants at equilibrium below pH 7 are given in Table 1 and show that, when ionization of citric acid was repressed by a lower pH value, greater concentrations of citrate and calcium were required to saturate the solu-

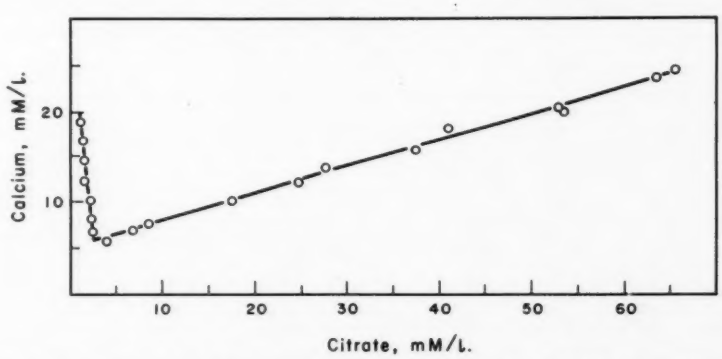

FIG. 1. Concentration of calcium and citrate at equilibrium, in solutions at or above pH 7 and at 21° C.

TABLE 1
Composition of supernatants at equilibrium below pH 7 and at 21 and 95° C.

Temp. ° C.	Initial pH	At equilibrium, concentrations in mM/liter					
		pH	Ca	Cit	Ca^{++}	$Cit^{\equiv}$	μ
			By precipitation[a]				
21	4.40	4.05	21.7	10.8	14.8	.027	.094
21	4.90	4.35	14.9	6.55	10.3	.035	.082
21	6.70	5.50	9.56	2.75	7.01	.066	.069
21	8.20	5.80	8.96	2.45	6.64	.068	.068
95	6.70	5.92	11.0	3.32	7.89	.095	.070
			By dissolution[b]				
21	.....	6.72	4.00	2.87	1.22	.090	.004
95	.....	6.52	3.12	2.02	1.17	.067	.004

[a] Initial solution contained 28.0 mM/liter calcium and 15.0 mM/liter citrate.
[b] Crystals of tricalcium citrate dissolved in water. Since K_s was not known at low ionic strength, calculations were made by Equation (1).

tion. However, as stated previously, the composition of the precipitate remained that of tricalcium citrate.

Solubility products $(Ca^{++})^3 \times (Cit^{\equiv})^2$ obtained at 21° C. varied with ionic strength. The relation between pk_s (−log. of solubility product) and $\sqrt{\mu}$ can be expressed as: $pk_s = (17.63 \pm 0.08) - (10.84 \pm 0.23)\sqrt{\mu}$ over the range of ionic strengths studied (Figure 2). The pk_s values for solutions at equilibrium

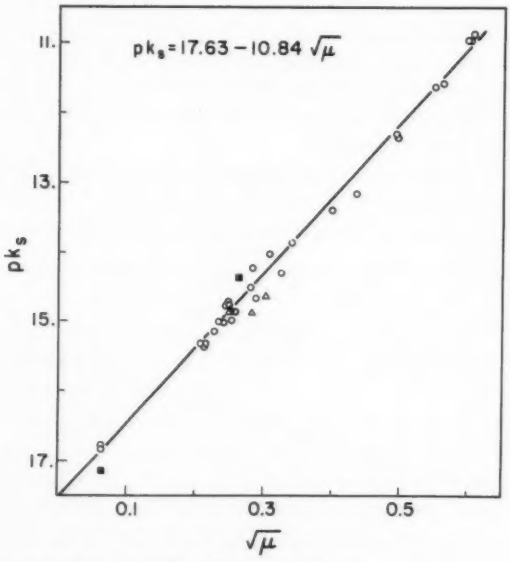

FIG. 2. Variation of pk_s value with $\sqrt{\mu}$ ionic stength:

○ pH > 7, 21° C. (data of Figure 1)
△ pH < 7, 21° C. (data of Table 1)
■ pH < 7, 95° C. (data of Table 1)

below pH 7 (μ 0.08) agreed within experimental variations with the value calculated for ionic strength 0.08. Also, solubility products calculated for supernatants obtained at 95° C. were not significantly different from those obtained at 21° C.

The presence of phosphate or magnesium in the calcium citrate solutions had no effect on the pk_s values, beyond those expected from the increased ionic strength (Tables 2 and 3). Addition of phosphate to the three saturated calcium citrate solutions did not measurably affect concentration either of total supernatant calcium and of citrate or Ca^{++} ion. The presence of magnesium, however, increased the level of Ca^{++} and decreased $Cit^{\equiv}$ concentration in all solutions studied. In two of the solutions, these changes were just compensating and, therefore, no other changes were required to maintain saturation. However, in the solution containing an excess of calcium over citrate, saturation was maintained by an increase in concentration of total calcium and citrate.

Milk ultrafiltrates. Concentrations of calcium and magnesium ions were calculated for ultrafiltrates prepared in this laboratory and for ultrafiltrates obtained by other workers (19, 23, 26, 28) and compared with the determined values (Table 4). The pH was not available for ultrafiltrates prepared by Smeets (26), but was assumed to be 6.77. Small errors in pH do not introduce large errors in calculating Ca^{++} and $Cit^{\equiv}$ concentrations. Even completely disregard-

TABLE 2

Effect of magnesium on solubility of calcium citrate at pH above 7 and at 21° C. (Concentrations in m*M*/liter)

Initial concentration		Mg. added	Concentrations at equilibrium						
Ca	Cit		Ca	Cit	Ca^{++}	Mg^{++}	$Cit^{\equiv}$	μ	pk_s[a]
18.5	12.0	Nil	5.53	4.01	1.81	.....	0.29	.044	14.77
18.5	12.0	3.0	7.03	4.33	4.07	1.74	0.10	.054	14.66
22.8	21.8	Nil	8.50	12.5	0.38	.....	4.38	.069	14.80
22.8	21.8	3.0	8.10	12.0	0.79	0.28	2.07	.075	14.67
30.0	38.0	Nil	13.4	27.7	0.57	.....	14.9	.162	14.77
30.0	38.0	3.0	13.4	27.3	0.71	0.16	11.8	.153	14.57

[a] pk_s expressed on the basis of ionic strength (μ) 0.08.

TABLE 3

Effect of phosphate on solubility of calcium citrate at pH's 6.8-7.0 and at 21° C. (Concentrations in m*M*/liter)

Initial concentrations[b]		P added	Concentrations at equilibrium					
Ca	Cit		Ca	Cit	Ca^{++}	$Cit^{\equiv}$	μ	pk_s[a]
6.45	3.7	Nil	idem	idem	2.92	.27	.054	14.26
6.45	3.7	3	6.55	3.67	2.74	.21	.058	14.52
8.35	12.3	Nil	idem	idem	0.45	4.40	.075	14.72
8.35	12.3	8	8.73	11.8	0.55	3.80	.085	14.79
12.6	26.8	Nil	idem	idem	0.55	14.7	.153	14.73
12.6	26.8	10	13.0	27.0	0.58	14.8	.165	14.86

[a] pk_s expressed on the basis of ionic strength (μ) 0.08.
[b] Solutions were already equiiibrated with tricalcium citrate before phosphate was added. Dilution introduced by addition of phosphate was of the order of 1%.

TABLE 4

Composition of milk ultrafiltrates

(Concentrations in m*M*/liter)

Sample	Determined							Calculated		
	pH	Ca	Mg	Cit	P	Ca^{++}	Mg^{++}	Ca^{++}	Mg^{++}	pk_a
1	6.77	7.77	2.73	8.34	14.5	2.7	.....	1.38	0.48	14.64
1 seeded	6.74	7.80	2.73	8.35	14.5	.....	.....	1.41	0.50	14.63
2	6.88	6.80	2.65	7.80	14.1	2.6	.....	1.16	0.45	14.78
2 seeded	6.72	6.44	2.84	7.77	14.1	.....	.....	1.18	0.52	14.81
3	6.87	9.74	2.86	8.72	9.81	.....	.....	2.49	0.72	14.22
White and Davies (28)						4.25	.....	3.53	1.32	14.16[a]
White and Davies (28)						3.78	.....	2.76	0.78	14.36
White and Davies (28)						2.90	.....	2.78	0.97	14.34
Smeets (26)						3.3	.....	2.58	0.80	14.18
Smeets (26)						2.3	.....	1.62	0.55	14.86[a]
Smeets (26)						4.4	.....	3.71	1.25	14.44
Rose and Tessier (23)						2.8	.....	1.82	0.63	14.47
Nordbo (19)						1.77	0.46	1.62	0.48	14.51
Nordbo (19)						2.06	0.48	1.62	0.47	14.41
Nordbo (19)						1.66	0.47	2.20	0.60	14.24
Kreveld and van Minnen (12)						1.8 to 3.0	0.65 to 1.10	.....	.....	.....
Christiansen *et al.* (3)						1.7 to 2.1	0.74 to 0.82	.....	.....	.....

[a] Differed significantly from statistically determined value for ionic strength 0.08. Seeded samples were agitated 67 hr. at 21° C., after seeding.

ing acid dissociations for an ultrafiltrate at pH 6.53 (i.e., assuming pH 7.0 or above), resulted in a $Cit^{\equiv}$ value that was only 3.8% high, a Ca^{++} that was 2% low, and a Mg^{++} that was 2.4% low but caused no change in pk_s value. The calculated Mg^{++} concentration varied with different ultrafiltrates and covered about the same range of values as the published data (3, 12, 19) summarized in Table 4. Calculated Ca^{++} concentrations were generally lower than the determined values, but the difference was not constant, indicating that the determined Ca^{++} concentration did not always follow changes in concentration of the complexing ions.

The ion product (pk_s) for the ultrafiltrates was obtained from the calculated Ca^{++} and $Cit^{\equiv}$ concentrations (Table 4) and it agreed in most cases with the values obtained with solutions of calcium citrate at μ 0.08 (Table 2). Also, agitation of two different milk ultrafiltrates with crystals of triclacium citrate at 21° C. for 67 hr. did not change their composition (1 seeded and 2 seeded, Table 4), indicating that these ultrafiltrates were already saturated with the salt. The two significant deviations may have been caused by differences in ionic strength or by the presence of unknown complexing agent(s). Actually, relatively small variations in ionic strength (from 0.11 to 0.07) could explain these variations.

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ULTRACENTRIFUGATION STUDIES OF MILK HEATED TO STERILIZATION TEMPERATURES [1]

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SUMMARY

The effect of heating milk on the sedimentation of protein particles was studied. Pictures taken of sediment during ultracentrifugation were measured to determine sedimentation constants in Svedberg units which were calculated for the last measured fractions of sediment thrown from solution at 10,000 G. Heating was done as follows: A. Milk was preheated at 180 ± 0.5° F. for 30 min., then in 2.08 sec. heated to 300 ± 0.5° F., and held for 2.08 sec., homogenized, and cooled. B. Same as A, except milk was sterilized at 285 ± 0.5° F. C. Milk was preheated to 180° F. in 2.08 sec., then heated to 300 ± 0.5° F. in 2.08 sec., and held for 2.08 sec., homogenized, and cooled. D. Same as C, except milk was sterilized at 285 ± 0.5° F. Constants for raw milk and the same milk with increasing heat treatments were, respectively, 440; 630; 844; 1,355; and 2,277 × 10^{-13}. Corresponding constants for the first half of sediment were 1,900; 2,700; 3,700; 3,800; and 6,600 × 10^{-13}. After storage at 40° F. for 72 days, the maximum volume of sediment was approximately 10% greater; whereas, the volume of sediment from the same milks stored at 70 and 100° F. the same length of time was less than the initial sediment. After storage for 71 days at 100° F., sedimentation constants for volumes of sediment equal to the respective volumes upon which constants for the first half of the sediment were calculated in freshly processed milks were 1,100, 1,300, 2,200, and 3,700 × 10^{-13}, indicating the degree of dissociation of the larger particles during storage.

Heating fluid milk products to high temperatures with a short or with no holding time is the present trend in the dairy industry. These high temperatures cause profound changes in the serum proteins of milk (5, 7). Only 7% of the serum proteins was removed from raw skimmilk by ultracentrifugation, as compared to 52% from milk heated to 242° F. for 2 min. (3). The whey proteins of raw milk also have been reported to be unaffected by ultracentrifugation (2). During storage less serum protein was removed by ultracentrifugation than was removed immediately after heating (9), indicating transient changes in the protein structure.

The casein particles in raw milk have been reported to be from 400 to 3,480 Å in diameter (4, 11), and larger ones with a sedimentation constant of 4,950 × 10^{-13} have been found in normal fresh milk (4). A casein monomer in dilute alkaline solution was reported to have a constant of 1.18 × 10^{-13} (11). The addition of calcium to milk increased sedimentation and removal of calcium by ion exchange decreased it (1). The difference between the noncasein nitrogen contents of raw and of heated milks was constant, regardless of the amount of protein removed by centrifugation (8).

The purpose of this investigation was to determine, immediately after heating and during storage, the physical stability of milk heated at increasing intensities by the ultra high–temperature short-time method. Physical stability was determined by subjecting milk to a centrifugal field.

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EXPERIMENTAL PROCEDURES

Fresh Grade A whole milk was obtained from the University Farm. It contained 4.2% fat and 9.14% solids-not-fat and had a pH of 6.70. It had a specific gravity of 1.0307 at 100, 1.0322 at 70, and 1.0341 at 40° F. The milk was heated in the Mallory small tube heat exchanger of which the preparative and operational characteristics have been described (6, 10).

The milk was processed as follows:

A. Milk was preheated in the Mallory heater from 50 to 180° F. in 2.08 sec., transferred to a vat and held for 30 min. at 180° F. It took 7.5 min. to heat 25 gal. of milk to 180° F. before it was held for 30 min. Then, immediately after the holding period, it took 7.5 min. more to sterilize this same volume of milk. Next, in 2.08 sec., the milk was heated to 300 ± 0.5° F., held for 2.08 sec., cooled to 150° F. in 2.08 sec., homogenized at 2,000 p.s.i.g. in the first stage and 500 p.s.i.g. in the second stage, and finally cooled in 4.16 sec. to 40 to 45° F.

B. The milk was processed as it was in A, except that it was heated to 285 ± 0.5° F.

C. Milk was preheated from 50 to 180° F. in 2.08 sec., then, in 2.08 sec. it was heated to 300 ± 0.5° F. and held for 2.08 sec., cooled and homogenized as it was in A.

D. The milk was processed the same as it was in C, except it was heated to 285 ± 0.5° F. for 2.08 sec.

E. Raw milk.

The sterilized milk samples were collected at the outlet of the Mallory heater in sterile screw-cap tubes which were stored at 40, 70, and 100° F.

These processed milks will be referred to in this paper as A, B, C, D, and E, in Figures 1, 2, 3, 4, and 5.

Centrifugation. The Fisher-Stern ultracentrifuge (12) was used. It has a plastic rotor which spins in a vertical plane and it was kept in a room which was controlled at 68 to 70° F. All milk samples, stored at 40 or 100° F., were held at 70° F. for 12 to 20 hr. to allow them to equilibrate, internally, before they were ultracentrifuged. The raw and sterile milks were ultracentrifuged within 2 to 4 hr. after sterilization. Pictures of the sediments taken during ultracentrifugation were measured from the negatives with a stereoscopic microscope, equipped with a filar micrometer eyepiece. The negatives were placed between two pieces of window glass which were clamped together. All sedimentations were photographed at 10,000 G.

RESULTS

The sedimentation curves for the raw milk E, and for the same milk after sterilization by four time-temperature combinations, A, B, C, and D, are shown in Figure 1. Sedimentation constants ($\times 10^{-13}$), calculated from Svedberg's equation ($S' = \frac{\Delta x}{\Delta t} \cdot \frac{1}{W^2 x}$) with Δt the time required to reach maximum depth

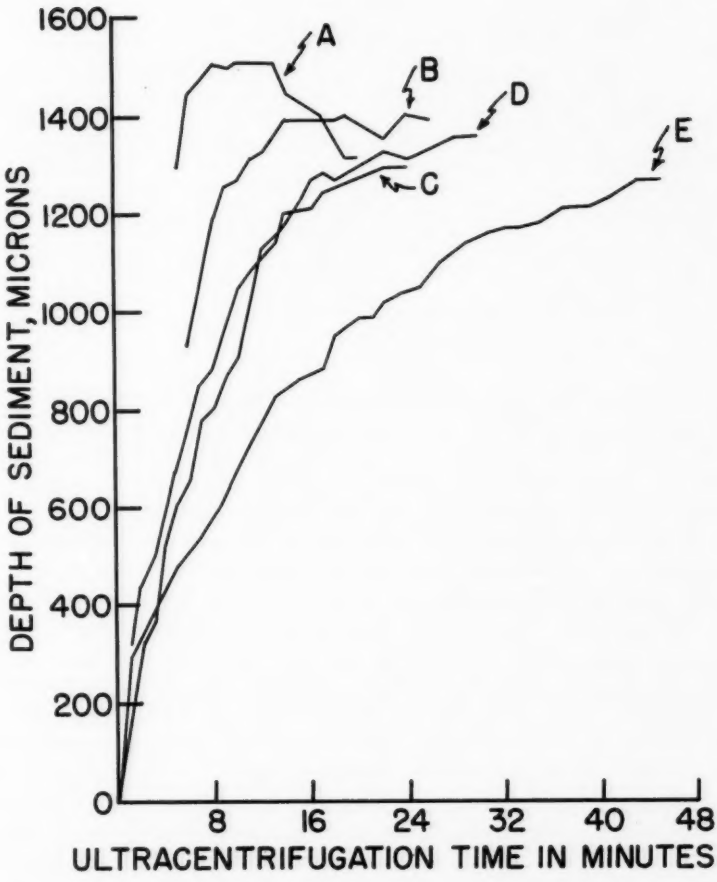

FIG. 1. Sedimentation rates for raw milk and for milk immediately after sterilization. Processing: A. Milk was preheated at 180 ± 0.5° F. for 30 min., sterilized at 300 ± 0.5° F. for 2.08 sec., homogenized, and cooled. B. Same as A, except milk was heated to 285 ± 0.5° F. for 2.08 sec. C. Milk was preheated to 180° F. in 2.08 sec., then to 300 ± 0.5° F. for 2.08 sec., homogenized, and cooled. D. Milk was processed the same it was in C, except it was heated to 285 ± 0.5° F. for 2.08 sec. E. Raw milk.

of sediment, were 2,277 for A, 1,355 for B, 844 for C, 630 for D, and 440 for E, the raw milk (Table 1). In other experiments not reported in this paper, milk heated to 140, 170, 180, 190, 200, 220, and 260° F. revealed curves between the one for raw milk and for milk heated at 285° F. and held for 2.08 sec. When milk was heated to 320 ± 0.5° F. with no holding time, the sedimentation curve was similar to the milk which was held at 180° F. for 30 min. and then sterilized at 300 ± 0.5° F. for 2.08 sec. (Curve A, Figure 1).

TABLE 1
Sedimentation constants at time of maximum depth of sediment

Heat treatment	2 to 4 hr. after processing	After storage at: 40° F. for 72 days	70° F. for 72 days	100° F. for 71 days
	($S \times 10^{-13}$)			
A. 180° F. for 30 min. 300° F. for 2.08 sec.	2,277	2,024	721	948
B. 180° F. for 30 min. 285° F. for 2.08 sec.	1,355	648	667	823
C. 180° F. for 2.08 sec. 300° F. for 2.08 sec.	844	757	632	665
D. 180° F. for 2.08 sec. 285° F. for 2.08 sec.	630	516	533	695
E. Raw milk	440			

The sedimentation curves for the different milks after storing them at 40° F. for 72 days are shown in Figure 2, including for reference the one for raw milk from Figure 1. Curve A represents a faster rate of sedimentation with more evidence of packing after the maximum sediment was obtained. Sedimentation constants ($\times 10^{-13}$), calculated at the time of maximum depth, were 2,024 for Curve A, 648 for B, 757 for C, and 516 for D. At the maximum depth of sediment for Curve A, the sedimentation constant was 2,024, but there was packing of the sediment followed by four peaks, the last of which had a sedimentation constant of 738. Less packing was observed in each of the other milks (Table 1).

Sedimentation curves after storing the milks for 72 and 71 days at 70 and 100° F., respectively, are shown in Figures 3 and 4. The sedimentation constants calculated at the time of maximum sediment were 721 for Curve A, 667 for B, 632 for C, and 533 for D at 70° F. storage, and they were 948 for A, 823 for B, 665 for C, and 695 for D at 100° F. storage (Table 1).

Differences between the curves in Figures 3 and 4, and the corresponding ones in Figure 1, are greatest for the large particles. Sedimentation constants for the first half of the sediment in Figure 1 were 6,600 for Curve A, 3,800 for B, 3,700 for C, 2,700 for D, and 1,900 for the raw milk. Constants for the same volume of sediment after storing the milks for 9, 22, and 71 days at 100° F. are shown in Figure 5.

DISCUSSION

The complex and variable nature of the proteins in milk has presented problems in studying their basic properties and changes that occur during heating. The ultracentrifuge used in this study (12) is adapted to sedimentation studies on milk proteins because of the short time, about 15 sec.,[2] required to reach the desired velocity, ability to photograph sedimentation, and accurate temperature control.

[2] A more efficient supply of air decreased the time from 25 sec. (12) to 15 sec. to reach the desired velocity.

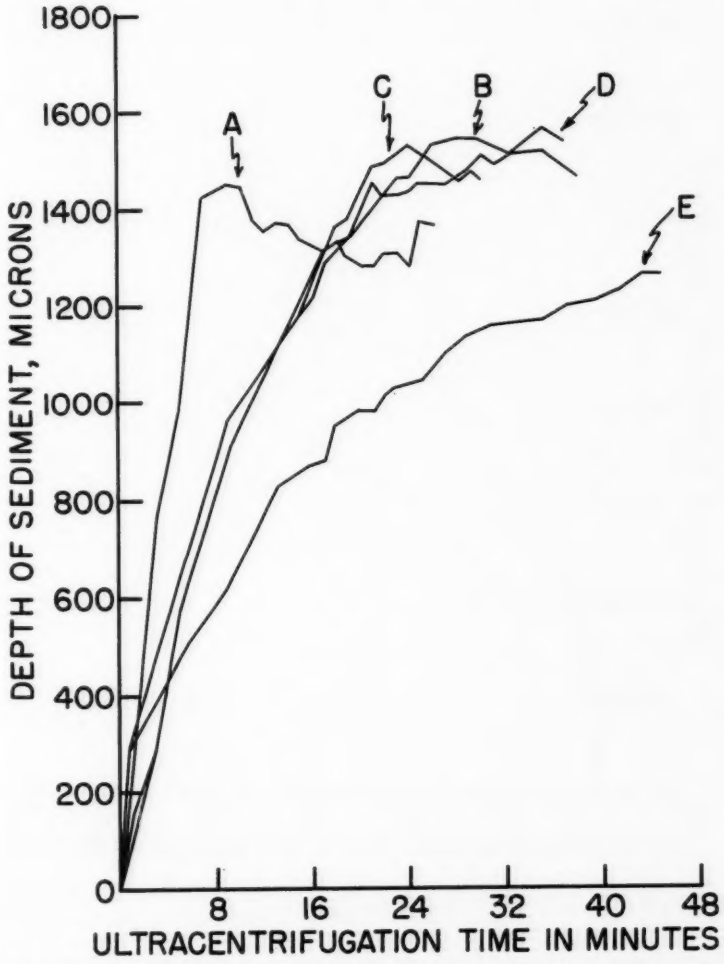

FIG. 2. Sedimentation rates for sterile milk after storage at 40° F. for 72 days. Processing for A, B, C, D, and E, the same as it was in Figure 1.

Analysis of variance of replicate runs indicated significant variations in the sedimentation curves due to the run. Explanations of the variations are the degree of accuracy within which the velocity of the ultracentrifuge could be controlled and difference in the exposure of individual pictures. However, similar points determining breaks in each of the replicate curves varied from their own best fitting smooth curve by more than the standard deviation of $\pm 31\ \mu$ for a single observation, including such sources of error as control of velocity and photography. The standard deviation was $\pm 9.8\ \mu$ for an individual measurement of a single picture.

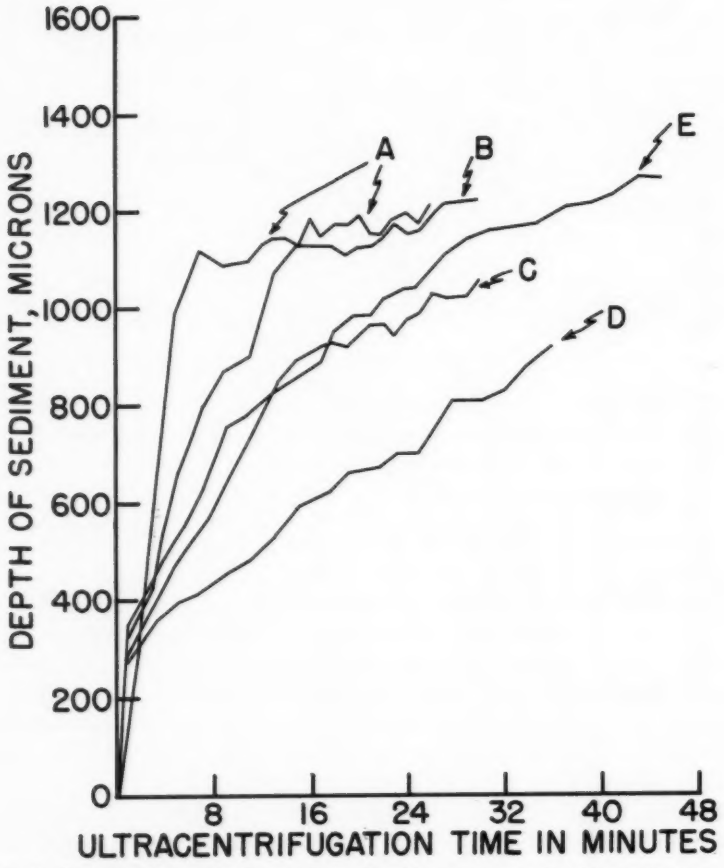

FIG. 3. Sedimentation rates for sterile milk after storage at 70° F. for 72 days. Processing for A, B, C, D, and E, the same as it was in Figure 1.

The sedimented protein from raw milk was transparent, but as the temperature of heating was increased, the amount of light transmitted through the sediment decreased. The sedimented protein was opaque in milks heated above 310° F. with a 2.08-sec. hold and in milks which had been held at 180° F. for 30 min. before final sterilization. Subsequent results have shown that fat may be partially responsible for this opacity. The maximum volume of sediment at 10,000 G increased immediately after processing. Packing of the sediment became more evident as the temperature of heating the milk was raised. The increase in volume of sediment, as a result of the addition of the denatured serum protein to the sedimented casein with higher heat treatments, was greater than expected. In addition to fat in the sediment, this suggested changes in the shape of the protein particles, the structure, or hydration.

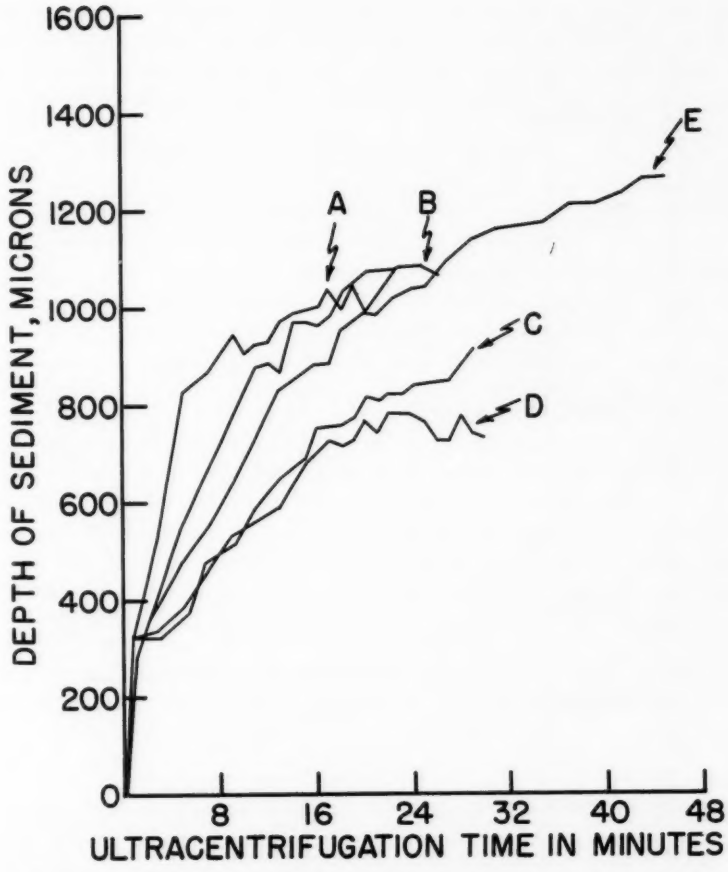

FIG. 4. Sedimentation rates for sterile milk after storage at 100° F. for 71 days. Processing for A, B, C, D, and E, the same as it was in Figure 1.

Ford and Ramsdell (4) concluded that casein particles in milk have definite sizes which are multiples of the smallest ones, and they gave sedimentation constants for the different sizes. Under the conditions of the present experiments, the sedimentation constants of Ford and Ramsdell for sizes of 3, 4, 6, 9, 12, and 16 units, correspond to sedimentation times of 46.6, 37.4, 29.3, 22.2, 18.9, and 15.0 min. Comparing these values with those in Figure 1, Curve E, for raw milk, revealed breaks at each of these time intervals which do not appear at other points on Curve E. Pictures were not taken at the proper time to show breaks for the larger particles, made up of 24, 32, 64, and 128 units. When pictures were taken of other ultracentrifuged raw milks, both small and large particles revealed characteristic sedimentation patterns and there was close agreement between the times calculated from the data of Ford and Ramsdell (4) and the

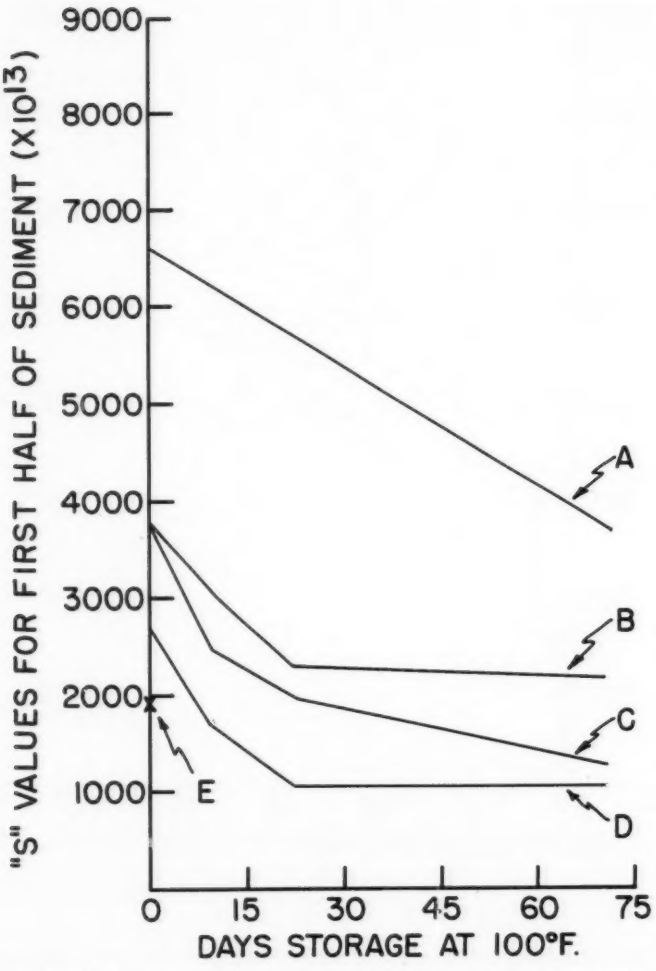

FIG. 5. Calculated sedimentation constants for volume of sediment equal to the first half of the maximum sediment from the freshly processed milks. Processing for A, B, C, D, and E, the same as it was in Figure 1.

breaks in the sedimentation curves obtained in our laboratory. When raw milk was clarified at 40 to 45° F., the sedimentation rate was faster and the curve was smoother than it was for the same milk before it was clarified. The greatest increase in sediment was in the range, S = 1,100, the size group that Ford and Ramsdell (4) considered to be predominant.

The shape of Curve E, Figure 1, is not what would be expected if the breaks or irregularities represented the last of each of the size groups that were sedi-

mented as reported by Ford and Ramsdell (4). This type of curve could be due to packing followed by further sedimenting, or it could be due to sedimenting bands formed by a sweeping action or by aggregating particles. It was observed after the cell had partially cleared, that bands of particles could be seen moving downward.

A comparison of the curves in Figure 1 indicates that the sedimentation rates increased with the higher temperatures of heating and that the changes above the temperature needed for sterilization were greater than the difference between raw milk and the minimum temperature used for sterilization.

Figures 2, 3, 4, and 5 show the effects of storage on the sedimentation of proteins and indicate that the dissociation of particles at 70° F. is similar to that at 100° F. but to a lesser degree. The increased maximum volume of sediment for 40° F. storage suggested hydration of the protein particles. The milk centrifuged and shown in Curve A, Figure 2, contained some sediment when it was removed from storage. It was mixed thoroughly before an aliquot was transferred to the ultracentrifuge cell. The sediment appeared to mix, but it is possible that it did not disperse sufficiently to yield a thoroughly representative sample. This could account for the lower volume of sediment.

Because of unexplained variations, the nitrogen analyses are not reported in detail but will be investigated further. However, it can be stated in general that neither the noncasein nor the nonprotein nitrogen increased in storage at 40° F. for 37 days. Both the noncasein and nonprotein nitrogen increased in the milks stored at 70 and 100° F. It appeared, subject to further investigation, that the increases in noncasein protein nitrogen and nonprotein nitrogen were greatest in milk which had been sterilized at the lower temperatures.

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A QUANTITATIVE TEST FOR CARRAGEENIN ESTER SULPHATE IN MILK PRODUCTS [1]

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SUMMARY

A method was developed to determine gravimetrically the concentration of carrageenin in some milk products by the sulphate content of a dialyzed milk-carrageenin system after hydrolysis with hydrochloric acid and decolorization with activated carbon. The effects of the variables involved were studied. The method was employed to determine the ester-sulphate content of the carrageenin anions of various salts of κ- and λ-carrageenin. The results obtained for the λ-carrageenin were in agreemnt with those predicted from the commonly accepted structure of this fraction, whereas the value for the κ-fraction suggested a slight alteration in its reported structure.

It is suggested that this method could also be used to determine the sulphate content of milk if the dialysis step were omitted.

Carrageenin, or Irish Moss extractive, is widely used in the dairy industry as a stabilizing agent in various products. It is a hydrocolloid extracted from the red sea plant, *Chondrus crispus* (4, 9), and the product is a heterogeneous mixture of polysaccharides with two main components, κ- and λ-carrageenin, both of which contain sulphate substituted galactose units (8). Interest in the interaction of carrageenin with milk proteins required the development of a suitable test for carrageenin in milk fractions. While the more common vegetable gums used in dairy products may be indicated and differentiated in milk on a qualitative basis (1), a quantitative analysis for these additives in milk has not been reported.

As carrageenin is characterized by its high content of ester-bound sulphate, an assay may conveniently be based upon this constituent. However, preliminary trials have shown that a common method for the estimation of ester sulphate in carrageenin (11) by the gravimetric determination of sulphate as barium sulphate in a nitric acid or a hydrochloric acid hydrolysate could not be applied to a carrageenin-milk system. In the removal of free sulphate ions prior to hydrolysis, the low pH conditions resulted in a removal of carrageenin with the coagulated proteins. Also a post-precipitation was observed in the nitric acid hydrolysate after the barium sulphate had been removed by filtration. The use of hydrochloric acid in the hydrolysis resulted in the formation of insoluble, dark-colored substances, which grossly interfered with the determination of sulphate. Preliminary studies showed, however, that such a hydrolysate could be clarified by the action of charcoal and filter aid. Therefore, this approach was investigated in the development of a quantitative determination of carrageenin ester sulphate in milk which has been shown to apply to some other dairy products.

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METHOD

Reagents

Hydrochloric acid (conc.): (36.5–38.0% A.R.)

Hydrochloric acid (1:20): 1 vol. of concentrated HCl is diluted with 19 vol. of distilled water.

Activated carbon: Darco G-60, Atlas Powder Co., Wilmington, Delaware, or other activated carbon of low sulphate content.

Filter aid: Sil-O-Cel, Johns-Manville Sales Corp., Chicago, Illinois, or other filter aid of low sulphate content.

Barium chloride (5% w/v): 5 g. of $BaCl_2$ (A.R.) is dissolved in 100 ml. of distilled water.

Procedure

Approximately 100 g. of milk weighed to the nearest gram is transferred to a 20–24 in. long Visking casing (No. 36/32) and the bag is closed so as to leave an air-filled headspace of not more than 25–30 ml., to prevent excessive dilution during dialysis. The sample is dialyzed in a cold room (5–6° C.) for a minimum of 48 hr. with 5–6 changes of precooled deionized water. After dialysis, the sample is transferred quantitatively to a 400-ml. beaker (calibrated with a 200-ml. mark). Ten milliliters of concentrated hydrochloric acid are added and the volume is made up to 200 ml. with deionized water. The beaker is covered and placed in a covered water bath at boiling temperature. After some hydrolysis, 8 g. of activated carbon and 2 g. of filter aid are added, and the hydrolysis is continued for a total of 6 hr. The mixture is then stirred thoroughly and the insoluble material is allowed to settle. The supernatant is decanted through Whatman No. 42 filter paper in a 9-cm. conical funnel. The precipitate in the beaker is suspended in 50 ml. hydrochloric acid (1:20) and heated in the water bath, and the supernatant decanted through the filter. The washing of the precipitate is repeated and, when the filter has drained dry, the contents of the beaker are transferred to the filter and washed once with approximately 50 ml. hydrochloric acid (1:20). The combined filtrates are collected in a 400-ml. beaker and placed on top of the water bath at 65–70° C. When hot, an excess of hot 5% barium chloride is added rapidly with agitation, and the beaker is covered and allowed to stand for at least 6 hr. at 65–70° C. The sample is then filtered through a tared Gooch crucible packed with acid-washed asbestos, and the beaker and crucible are washed with 100 ml. of deionized water. A few drops of barium chloride are added to the filtrate, which is held for a few hours to insure complete removal of sulphate, and refiltered if necessary. In some instances, especially when the sulphate content is of the magnitude of only a few milligrams, post-precipitation of barium sulphate may occur after the passage through the Gooch crucible. This condition may be prevented by allowing a longer time for crystal growth if convenient. In this connection, it is advantageous to use the same crucibles for successive runs and not prepare fresh ones, since they would provide nuclei for retention of the last traces of barium sulphate in the filtrate. The crucible is ignited at 900° C. and weighed after cooling to room temperature

in a desiccator. The increase in weight is recorded as barium sulphate. Unless the sulphate content of the reagents is known, it is necessary to determine a reagent blank by the same procedure. The content of ester sulphate in the sample is calculated as follows:

$$\% \text{ ester sulphate} = \frac{(\text{wt. of } BaSO_4 \text{ in sample} - \text{wt. of } BaSO_4 \text{ in blank}) \times 41.15}{\text{wt. of sample}}$$

A modification of the above procedure is possible if a control without added carrageenin, but otherwise of the same identity as the sample, is available. In this case, the dialysis step may be omitted and, instead, the sample and the corresponding control may be hydrolyzed immediately with concentrated hydrochloric acid in the proportion of 1:20. The results in this case represent the total sulphate content of the carrageenin.

EFFECT OF VARIABLES

In investigating the effect of the variables involved, the modified procedure was used except where the dialysis step was specifically called for, to avoid the variability among different milk samples. This omission would not have any serious effect on the conclusions drawn, except that the results obtained would represent the total sulphate content in carrageenin and not just the ester sulphate.

The carrageenin used in these studies was Seakem Type 7, Lot 171105, supplied by Seaplant Corporation, New Bedford, Massachusetts. The carrageenin dispersions were prepared by first warming a known weight of distilled water or commercial, homogenized, pasteurized whole milk to 50–60° C. in a 600-ml. beaker and adding the powder slowly under rapid agitation with a mechanical stirrer. The agitation was continued 15 min., or until all of the material was dissolved. The viscous liquid was then transferred quantitatively to a glass-stoppered flask and, after cooling, adjusted to the desired volume or concentration. In these experiments, no effort was made to control the moisture content of the carrageenin samples employed, except that it was constant within each experiment.

The data for each experiment were obtained in duplicate and examined by an analysis of variance. A typical table of data and its analysis is given in Table 1, and the results of the remaining experiments investigating the effects of the several variables in the test are summarized in Table 2.

Amounts of activated carbon and filter aid. During acid hydrolysis, the samples turn dark-brown and contain insoluble substances. Upon the addition of activated carbon and filter aid, followed by filtration, a clear filtrate can be obtained. The control necessary in weighing the activated carbon and filter aid was investigated at three levels of activated carbon: 4, 8, and 12 g., and two levels of filter aid: 2 and 4 g. The results are reported in Table 1. Analysis of variance of these results indicates a significant contribution of sulphate by the activated carbon as well as the filter aid. These effects are independent of the presence of carrageenin, as shown by the lack of significance of the various cross

TABLE 1
Effect of activated carbon and filter aid upon the sulphate content of milk-carrageenin systems

Weight of activated carbon	Weight of filter aid	Sulphate content (duplicates)		Sulphate content of carrageenin
		Milk with 0.0969% carrageenin	Control milk	
(g.)	*(g.)*	*(mg/100 g.)*	*(mg/100 g.)*	*(%)*
4	2	38.29	12.37	26.78
	4	39.46	13.94	26.36
8	2	41.94	16.38	26.40
	4	42.44	16.84	26.43
12	2	43.98	19.24	25.56
	4	44.48	19.20	26.10
			Mean:	26.27 ± 0.22

Analysis of variance of sulphate content

Source of variance	Degrees of freedom	Sum of squares	Mean squares	Variance ratio	Significance[a]
Carrageenin (C)	1	3,880.872	3,880.872	14,267.91	V.S.
Filter aid	1	2.849	2.849	10.47	1%
Activated carbon (Ch)	(2)	(132.150)	66.075	243.00	1%
Linear	1	130.645	130.645	480.00	1%
Quadratic	1	1.505	1.505	5.53	5%
C × F	1	0.005	0.005	0.02	N.S.
C × Ch	2	0.570	0.285	1.05	N.S.
Ch × F	2	1.435	0.718	2.64	N.S.
C × F × Ch	2	0.216	0.108	0.40	N.S.
Error	12	3.266	0.272		
Total	23	4,021.363			

[a] V.S. = very significant; N.S. = not significant.

products with carrageenin and, therefore, the sulphate content of the carrageenin is unaffected when calculated as the difference between a sample and a corresponding control. By ignoring a quadratic response significant at the 5% confidence level, the effect of activated carbon levels can be satisfactorily explained by a linear relationship between the sulphate content and the amount of activated carbon. From the slope of the linear least-squares equation and error variance, it was estimated that the activated carbon should be weighed with an accuracy of at least ± 0.37 g., in order to contribute not more than one-fourth of the error variance.

Since the filter aid was shown to be a significant source of variance in the analysis, its effect upon the test was investigated more completely in another experiment. Four levels of filter aid: 0, 2, 4, and 6 g., at a constant weight of activated carbon, were investigated and the analysis of variance showed that there is a linear relationship between the sulphate content and the amount of

TABLE 2

Effect of variables

Variable	Investigated levels	Source of significant variance[a] in terms of: mg. % $SO_4^=$ in sample		% $SO_4^=$ in carrageenin	Selected value	Control necessary
Amount of activated carbon *(g.)*	4 8 12	Presence of carrageenin Carbon: Linear response[b] Quadratic response	(1%) (1%) (5%)	None	8 g.	± 0.37 g.
Amount of filter aid *(g.)*	0.0 2.0 4.0 6.0	Presence of carrageenin Filter aid: Linear response[c]	(1%) (1%)	None	2 g.	± 1.05 g.
Types of activated carbon	Darco G-60 Darco S-51 Nuchar C-190A Nuchar C-1000N	Presence of carrageenin Type	(1%) (1%)	None	Darco G-60	Low sulphate content is recommended
Hydrolysis time *(hr.)*	4 6 8 12	Presence of carrageenin Hydrolysis time: 4 vs. 6, 8, 10	(1%) (1%)	Hydrolysis time: 4 vs. 6, 8, 10	6 hr.	At least 6 hr.
Final volume of hydrolysis mixture *(ml.)*	150 200	Presence of carrageenin	(1%)		200 ml.	Not important
Final acid strength of hydrolysis mixture (Volume of conc. HCl: final volume)	0.8:20 1.0:20 1.2:20	Final acid strength: 0.8:20 vs. higher concentrations	 (1%)	None	1.0:20	At least 1.0:20
Dialysis	None 40 hr.	.....		Dialysis (5%)	Dialysis	Exhaustive
pH during dialysis	3 4 5 6 7	.....		Dialysis (1%)	Sample pH	Not important in the investigated range

[a] All other sources of variance were found not to be significant.
[b] Linear regression coefficient: 0.71 mg. $SO_4^=$ per gram of carbon.
[c] Linear regression coefficient: 0.48 mg. $SO_4^=$ per gram of Filter Aid.

filter aid. From the slope of the least-squares equation and the error variance, it was estimated that the filter aid should be weighed with an accuracy of at least ± 1.05 g., in order to contribute not more than one-fourth of the error variance.

Type of activated carbon. To study the effect of different types of activated carbon in the test, an experiment was performed in which four different types of activated carbon were investigated. The analysis of variance indicated that the amounts of sulphate in the different types of activated carbon vary greatly. Although the interaction term was not significant, it was considered desirable to use an activated carbon with a low sulphate content, for example, Darco G-60.

Hydrolysis time. Since, in the procedures currently in use for carrageenin ester sulphate variable times of hydrolysis are employed, the influence of this variable upon the results of the test was investigated for the following times: 4, 6, 8, and 16 hr. It was found that, for 4 hr. of hydrolysis, low sulphate values were obtained, while there were no significant differences between the results after hydrolysis for longer time intervals. However, it has been observed from subsequent studies that the filtration and washing procedures in the test are appreciably easier when a prolonged hydrolysis has been used. This is especially true when dealing with ice cream mix.

Dilution of the samples and strength of acid used for hydrolysis. The concentration of solids in the milk-carrageenin system and the concentration of acid during hydrolysis may be expected to influence the results of the test. To determine the control necessary, the effect of variation in these two factors was investigated for three final strengths of acid: 0.8, 1.0, and 1.2 ml. of hydrochloric acid per 20 ml. of hydrolysis mixture, and for two final volumes: 150 and 200 ml. Analysis of the data indicated that a final acid strength of 0.8 ml. of concentrated hydrochloric acid per 20 ml. of hydrolysis mixture gives significantly lower values than the higher concentrations. However, it was shown that even under these conditions the results of the determinations will not be affected, provided the final acid strength is the same in sample and control. The effect of dilution of the samples was not significant.

Dialysis against water. Since the carrageenin employed may contain appreciable inorganic sulphate, the true measure of the carrageenin content of the sample is the ester sulphate content rather than the total sulphate, provided the milk product itself is free of ester sulphate. To verify this and to establish the conditions for this treatment, 0.05 and 0.10% carrageenin systems in milk and water were dialyzed against tenfold volumes of deionized water, as indicated in the procedure. The progress of the dialysis was followed by conductivity measurements of the water after each change to insure an exhaustive treatment. It was apparent from these measurements that this was achieved in 40 hr. of dialysis. Therefore, the 48-hr. dialysis proposed in the procedure provides a safety margin of reasonable magnitude.

After dialysis, the samples were brought to room temperature and the pH was measured; the values are reported in Table 3. It will be seen from these values that upon dialysis of carrageenin dispersions against water, a measurable

TABLE 3

Effect of exhaustive dialysis against water upon the pH of milk-carrageenin systems

Dispersing medium	Carrageenin concentration (%)	pH	
		Undialyzed	Dialyzed
Milk	0	6.67	6.58
	0.0495	6.64	6.49
	0.1003	6.60	6.00
Water	0.0502	6.85	6.20
	0.1004	7.15	5.50

drop in pH takes place. This effect becomes pronounced with increasing concentration of carrageenin. However, milk has a sufficient buffer capacity to minimize this effect. A significance at the 1% confidence level of the dialysis treatment indicated the possible presence of free sulphate in the carrageenin or hydrolysis due to the lowering of the pH during dialysis.

Dialysis against buffered media at varying pH. The change in pH during dialysis apparently has been caused by membrane hydrolysis (5), which arises from the requirement that an over-all electrical neutrality must be maintained. To investigate whether the observed pH drop may cause some hydrolysis of the ester sulphate and to establish the control necessary, analysis for sulphate was performed on a 0.10% aqueous dispersion of carrageenin after dialysis against buffered media of ionic strength 0.05 under varying pH conditions. The samples were dialyzed for 40 hr. at 6° C. with five changes of dialysis medium. Analysis of variance of the results indicated that within the pH limits investigated no differences occur in the ester-sulphate content of carrageenin. Therefore, no hydrolysis takes place during dialysis, even at pH values as low as 3.0. It was noted that the carrageenin employed contained a significant amount of dialyzable sulphate ($1.61 \pm 0.24\%$ $SO_4^=$).

Recovery and reproducibility. To establish the recovery and reproducibility of the test, and to explore its usefulness in the carrageenin analysis of some other milk products, where carrageenin frequently may be expected, samples of water, milk, chocolate milk, and ice cream mix (diluted and undiluted), containing approximately 0.03 and 0.10% carrageenin, were tested. The results are reported in Table 4. Analysis of variance showed that there are no significant differences between the various samples or concentration levels, and the recovery of carrageenin ester sulphate in the various products at the different concentrations is complete within experimental error. The reproducibility expressed by the standard deviation calculated from the error variance is $\pm$ 0.60 mg. % $SO_4^=$.

SULPHATE CONTENT OF VARIOUS SALTS OF κ- AND λ-CARRAGEENIN

To further substantiate the validity of the test for carrageenin ester sulphate, a number of metal salts of κ- and λ-carrageenin were analyzed for the major cations and anions, moisture, and total sulphate, and the ester sulphate content of the carrageenin anions calculated to show whether or not the test yields the

expected value for their sulphate content. The moisture content was determined by drying 0.5–1.0 g. of the sample in vacuum at temperatures below 100° C. The sodium, potassium, and calcium contents were determined by a flame photometer (Perkin Elmer Model 52C), according to the internal standard method described by Murthy and Whitney (7). The sum of calcium and magnesium was determined by titration with versenate by a modification of the procedure of Jenness (6). For this determination, the samples were prepared by hydrolyzing the

TABLE 4

Recovery of carrageenin ester sulphate from various milk products (duplicates)

Sample	Concentration of carrageenin	Ester sulphate in sample	Recovery[a]
	(%)	*(mg. %)*	*(%)*
Water	0.0000	0.00	
	0.0295	6.85	97.3 ± 8.5
	0.0984	23.10	98.6 ± 2.6
Whole homogenized milk: commercial sample	0.0000	−0.25	
	0.0295	7.25	103.0 ± 8.5
	0.0984	24.10	102.9 ± 2.6
Chocolate milk: 2% fat 0.85% cocoa 5.0% sucrose 8.0% m.s.n.f.	0.0000	−0.70	
	0.0295	6.95	98.7 ± 8.5
	0.0984	23.80	101.6 ± 2.6
Ice cream mix: 12.5% fat 10.0% m.s.n.f. 16.0% sucrose 0.5% egg yolk	0.0000	0.10	
	0.0295	6.75	95.9 ± 8.5
	0.0984	24.30	103.8 ± 2.6
Ice cream mix: Same as above, diluted with an equal volume of water	0.0000	0.30	
	0.0295	6.35	90.2 ± 8.5
	0.0984	24.00	102.5 ± 2.6
		Standard deviation ± 0.60 mg. % $SO_4^=$	

[a] Ester sulphate content of this carrageenin sample was established by eight independent determinations of water dispersions (0.10%).

Mean: 23.80% $SO_4^=$

Standard deviation of the mean: ± 0.25% $SO_4^=$

carrageenin with acid, adjusting pH to 8–10, adding the borate buffer, and clarifying the solution with activated carbon and filter aid. The titration data were corrected for the presence of calcium in the filter aid and for its adsorption by the charcoal. Phosphate was determined by the method of Fiske and Subbarow (3) and the ion species present were calculated on the basis of the pH determination. Chloride was determined by the method of Davies (2). Bicarbonate was determined by titration with 0.05 *N* HCl to pH 3.3 and correcting for the presence of the $HPO_4^=$ ion. Total sulphate was determined by the proposed method without dialysis. The results are reported in Table 5.

TABLE 5

Composition of ion balance of salts of carrageenin fractions (wet weight basis)

Fraction	Salt	Moisture	pH	Cations				Anions					Ion balance		Ester sulphate in anion[b]
				Na^+	K^+	Mg^{++}	Ca^{++}	$HPO_4^=$	$H_2PO_4^-$	Cl^-	HCO_3^-	RSO_4^- [a]	Total cation	Total anion	
		(%)		(meq/100 g.)											(%)
λ	Na	10.11	6.67	224.9	136.3	6.4	1.8	0.7	1.2	0.0	11.8	336.9	369.4	350.8	39.92
	K	8.93	6.31	0.0	352.1	16.1	1.4	0.3	1.2	0.0	15.5	327.2	369.6	344.2	39.42
	Mg	12.34	6.46	0.0	174.1	166.2	1.4	0.4	1.0	2.0	13.3	322.7	341.7	339.4	39.68
	Ca	12.16	6.39	0.0	138.6	8.8	214.9	0.3	1.1	1.5	9.4	326.9	362.3	339.2	38.89
														Mean:	39.5 ± 0.22
														Expected from Figure 1(a):	39.83
κ	Na	12.34	6.22	101.9	99.0	27.9	22.4	0.1	0.7	4.0	13.1	233.5	251.2	251.4	28.16
	K	10.94	6.51	0.0	241.3	9.4	1.4	0.2	0.6	5.5	20.7	231.0	251.1	258.0	28.46
	Mg	13.72	6.18	0.0	92.8	137.7	1.4	0.1	0.7	8.0	10.1	224.2	231.9	243.1	26.93
	Ca	13.44	6.27	0.0	82.7	0.0	164.4	0.1	0.6	13.5	8.6	225.3	247.1	248.1	27.40
														Mean:	27.7 ± 0.35
														Expected from Figure 1(b):	29.93
														Expected from Figure 1(c):	27.78

[a] In this analysis, the values for sulphate have been determined as total sulphate.

[b] In the case of the λ-fraction these values have been calculated assuming the difference in the ion balance was due to the presence of free $SO_4^=$.

DISCUSSION

The investigation of the effect of the variables involved in the test indicates that, as long as a reagent blank is treated in a manner similar to that of the sample, accurate determinations of their ester-sulphate content can be obtained. Because of the presence of sulphate in the activated carbon and filter aid employed, it is necessary to control the weights of these reagents to $\pm$ 0.37 and $\pm$ 1.05 g., respectively. However, it should be emphasized that these limits apply to the particular activated carbon and filter aid employed in this study and would, of course, be expected to vary with other lots of these materials. Minima of 6 hr. of hydrolysis time and a final normality before hydrolysis of 0.5 *N* HCl appear to be desirable. Exhaustive dialysis of a carrageenin dispersion results in a drop in pH in the sample due to membrane hydrolysis, but it was shown that a pH of 3 during dialysis did not hydrolyze the ester-sulphate bonds.

The recoveries of carrageenin ester sulphate from milk products compared with the recoveries from aqueous dispersions are not significantly different. The test is apparently satisfactorily quantitative over the range investigated. However, while the adequacy of any analytical test may be established through the amounts recovered, and the reproducibility may be expressed by the error variance, the recovery itself is influenced by: (a) the reproducibility of the individual determinations (error variance); (b) the number of replications from which the mean has been calculated; and (c) the sample size employed. The accuracy of a test, therefore, may best be judged from the uncertainty associated with the recovery determination. For this analysis, in which the determination is based on the difference in concentration of a sample and a blank, it may be shown that the uncertainty in the percentage recovery may be expressed by the following equation:

$$S_R^2 = 20{,}000 \frac{S_Y^2}{n \times A^2}, \text{ where}$$

S_R = Standard deviation of the mean percentage recovery

S_Y = Standard deviation of the individual determinations ($\pm$ 0.60 mg. % $SO_4^=$)

n = Number of replicate determinations

A = Actual concentration (mg. % $SO_4^=$)

From this equation, it is possible to calculate the uncertainty in the recovery of sulphate for any concentration level and any number of replicates.

The composition and the ion balance of the various salts of the carrageenin fractions indicated the validity of the test (Table 5). It was shown by a Student's "t" test for paired observations of the total cations and anions observed in the κ-fraction that the difference was not statistically significant; whereas the anion content of the λ-fraction was significantly lower than the cation content. By reasoning from the method by which the carrageenin was fractionated (11), it was assumed that this difference was due to free sulphate, and it was shown by a Student's "t" test that the sulphate content of the carrageenin anion, calcu-

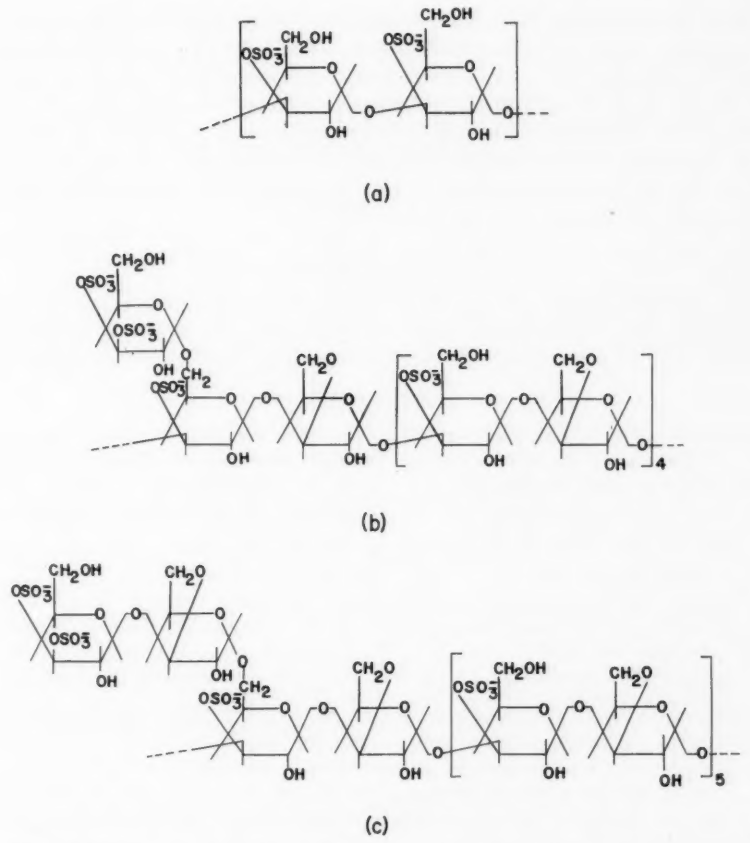

FIG. 1. Structure of the repeating unit of the carrageenin anions: (a) λ-carrageenin reported by Smith *et al.* (10); (b) κ-carrageenin reported by O'Neill (8); (c) possible modification for κ-carrageenin.

lated on this basis, did not differ significantly from that expected from the reported structure of λ-carrageenin (10) [Figure 1(a)]. In the case of the κ-fraction, the sulphate content of the anion was significantly lower than the calculated value for the reported structure by O'Neill (8) [Figure 1(b)]. However, the value was not different from the analytical value that he reported. Therefore, a possible structure is suggested [Figure 1(c)] which accounts for the experimental values. On this basis, the ratio of sulphated to nonsulphated residues in the κ-carrageenin is 1:1 rather than the reported 1.2:1. It is of interest that O'Neill observed a ratio of 1.1:1 on the basis of the weights of the isolated mercaptals.

The test is suitable for routine analysis of many samples and does not require special apparatus. The method may be adopted as a test for sulphate in milk, since it may be seen from Table 4 that the sulphate content in the dialyzed milk sample is the same within experimental error as that obtained in the reagent blank or, in other words, all of the sulphate present in milk is in the dialyzable form. Therefore, if the test is performed upon undialyzed milk, its sulphate content can be determined. An analysis of a milk sample performed in this manner indicated a sulphate content of 11.7 mg. %.

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ROLE OF LEUCOCYTES IN THE REDUCTION OF RESAZURIN IN RAW MILK [1]

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SUMMARY

Leucocytes, freshly isolated from bovine blood, rapidly reduced resazurin in raw milk, but these leucocytes rapidly lost activity on storage in milk at 37° C. Leucocytes isolated from raw milk had only a weak ability to reduce resazurin, and removal of leucocytes from raw milk by centrifugation had little influence on the reducing activities of the milk. Incubation of leucocytes, isolated from raw milk, with fresh bovine plasma resulted in a reactivation of the reducing powers of the leucocytes. Disintegrated leucocytes did not contribute substrates for the reducing systems of raw milk.

In an effort to determine the agents in raw milk which contribute to the reduction of resazurin, work was initiated on the role of leucocytes in this process. McBride and Golding (4) found a highly significant correlation between resazurin reduction of freshly drawn mastitic-like milk and the confirmatory test used. Little (3) observed that samples rapidly reduced resazurin to purple-pink, but would not reduce methylene blue until late in the incubation period and, in many cases, not at all. The work of these and other workers has resulted in the generally accepted view that a leucocyte count of greater than 500,000 per milliliter is indicative of a mastitic infection. Thus, McBride and Golding (4) and also Davis (1) state, unequivocally, that if the resazurin test is performed on milk within 4 hr. of milking, the reduction is a measure of leucocyte activity. Although the correlation of reduction times with numbers of leucocytes is of value, it must be remembered that high leucocyte counts also may be related to a high level of oxidizing enzymes in milk and to a high level of substances which may be unusually active as hydrogen donors. It does not necessarily follow, therefore, that the leucocytes are the actual agents carrying out the reduction process.

There have been only a few attempts to determine the reducing activity of leucocytes by adding them to milk containing an oxidation-reduction dye. Skar (6) demonstrated the reduction of methylene blue in sterile milk by leucocytes obtained from the lymph gland of a steer. He contended that a leucocyte count of 6.7 million per milliliter could not effect reduction in the standard test, but that they could reduce the dye if they were kept evenly distributed by periodic agitation of the milk during incubation. Strynadka and Thornton (7) have indicated that Barthel also recognized the reducing power of leucocytes, although he attributed little importance to this phenomenon in milk control. Strynadka and Thornton obtained blood from the jugular vein of a cow and separated the leucocytes by repeated centrifugation and washing

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with physiological saline. They were unable to show any significant alteration in reduction time of methylene blue when saline suspensions of leucocytes were added to samples of raw milk. They also found that the coefficient of correlation between leucocyte counts and the standard methylene blue reduction times of 158 samples was not sufficiently high to "justify the popular assumption that leucocytes reduce methylene blue in milk." Nilsson (5) was unable to obtain any change in E_h in samples of pasteurized milk to which lysed or washed leucocytes had been added. Unfortunately, Nilsson washed the leucocytes in sterile water rather than physiological saline.

In the present work, leucocytes from bovine blood and from milk were added to various samples of raw and pasteurized milk and the changes in resazurin reduction times noted. Also, efforts were made to determine whether sonic extracts of leucocytes would reduce resazurin and whether constituents of the leucocytes would act as active hydrogen donors for resazurin reduction.

METHODS

The blood was collected directly from the jugular vein of a steer as it was severed at the slaughterhouse. The blood was mixed in a ratio of 4:1 with a sterilized anticoagulant solution containing 13.2 g. anhydrous sodium citrate and 4.9 g. citric acid per liter. The blood was centrifuged at $1,100 \times G$ for 15 min. and the plasma removed. The uppermost layer of cells containing the leucocytes was then removed, resuspended in physiological saline, recentrifuged at $1,100 \times G$ for 15 min., and the process repeated until a suspension of leucocytes essentially free of red cells was obtained. Cell counts were carried out with the aid of a hemocytometer. Ribonuclease and desoxyribonuclease were the products of Worthington Biochemicals Corporation.

RESULTS AND DISCUSSION

To establish whether the mere addition of leucocytes would have an influence on the time required to reduce resazurin, various concentrations of washed leucocytes were added to raw milk taken from individual cows. A pronounced decrease in reduction time was achieved by the addition of the leucocytes and it was established that a concentration of 1,000,000 per milliliter would reduce resazurin to the 7/4 standard within 20 min.

It is evident from the data of Table 1 that leucocytes from bovine blood have a strong ability to reduce resazurin. It does not follow, however, that

TABLE 1

Influence of added leucocytes on time required for resazurin reduction

Cow No.	Milk	Milk $+10^6$ leucocytes/ml
	(min.)	
1	490	15
2	435	15
3	390	20
4	170	20

leucocytes, as they occur in raw milk, contribute to the reduction of resazurin. To check on this, samples of normal raw milk containing from 50,000 to 400,000 leucocytes per milliliter and from a few hundred to, in one case, 20,000 bacteria per milliliter, were centrifuged at 1,100 × G for 15 min. in a refrigerated centrifuge. Under these conditions, the fat was a firm layer at the surface and was easily removed with a spatula. The leucocytes and bacteria formed a pellet at the bottom of the tube. When the centrifuged milk was examined microscopically, no evidence of leucocytes could be found in the fluid skim layer and the plate count showed that only a few hundred bacteria per milliliter were present. It was, therefore, possible to pipette off the skimmilk from the pellet and thus determine the contribution of the bacteria and leucocytes to resazurin reduction. In each case reported, the fat was reincorporated by treatment with a Teflon Van Potter homogenizer for about 5 min. (Table 2).

TABLE 2

Influence of removal of leucocytes and bacteria from milk on the rate of resazurin reduction

Cow No.	Milk	Milk minus bacteria and leucocytes
	(min.)	
5	165	165
6	190	195
7	205	210
8	90	120
9	270	330

These data would indicate that, in the milks being studied, the bacteria and leucocytes do not have an important influence on the rate of resazurin reduction. Further information on the reducing activities of leucocytes was obtained by separating leucocytes from milk of high leucocyte and low bacterial content. The leucocytes were washed either in normal milk or in physiological saline and added to the raw milk of individual cows to give a final concentration of about 4,000,000 cells per milliliter of milk. The two systems of washing the leucocytes were used in an effort to confirm the report of Johns (2), that leucocytes washed in physiological saline lost their ability to reduce resazurin, whereas those washed in normal milk retained their reducing activities. We were unable to confirm this observation but did find that, when added at this higher concentration, the leucocytes isolated from milk did increase the rate of reduction (Table 3).

TABLE 3

Ability of leucocytes recovered from milk to reduce resazurin

Cow No.	Reduction times (min.)				
	11	12	13	14	15
a. Milk	300	470	250	180	285
b. a + leucocytes washed in milk	215	275	170	150	220
c. a + leucocytes washed in saline	....	295	195	165	

However, one must conclude that, since a final concentration of 4,000,000 leucocytes per milliliter was required to produce these changes in reduction time, and since the removal of leucocytes and bacteria by centrifugation had a negligible effect on the reduction time, leucocytes as they occur in milk at concentrations of 500,000 to 1,000,000 or more are not responsible for marked increases in the rate of reduction of resazurin.

The fact that leucocytes isolated from bovine blood were so much more active than those separated from milk led us to try to simulate, in a very crude fashion, the conditions under which the leucocytes occur in the udder. Freshly isolated leucocytes from bovine blood were held in milk at 37° C. for 4 hr., resazurin was then added, and the reduction time determined (Table 4).

TABLE 4

Influence of storage on reducing activities of leucocytes

	Reduction times (min.)			
Cow No.	16	17	18	19
a. Milk	530	390	390	100
b. Milk + fresh leucocytes	20	20	20	35
c. b incubated at 37° C. for 4 hr. before addition of resazurin	215	150	140	60
d. Milk + leucocytes refrigerated in saline 24 hr.	110	75	75	....
Bacterial count of milk	300	600	500	700
Bacterial count after 4 hr. of incubation at 37° C.	5,000	2,000	43,000	2,000

Longer incubation times were avoided, due to the possibility of complicating the picture by the presence of large numbers of bacteria. Storage of the leucocytes in physiological saline for 24 hr. at 4° C. also markedly lessened their activity. This rapid loss of activity should mean that, even in milks where the bacterial content is constant, the contribution to the rate of reduction by leucocytes decreases rapidly as the milk is held.

Milks which are Grade B by virtue of their leucocyte content will certainly improve markedly on storage. Another interpretation of the change in reducing activities of leucocytes on storage is that the changes actually occurred in the milk rather than in the leucocytes. If the hydrogen donors available to the leucocytes were limiting, then old samples of milk conceivably could be low in these essential compounds. However, the addition of suspensions of fresh leucocytes to a variety of milks of different ages and also to pasteurized milk revealed that the age of the milk had no influence on the rate at which fresh leucocytes reduced resazurin.

When leucocytes separated from milk were suspended in fresh bovine plasma, it was found that within 2 hr. they regained much of the reducing activity shown by those freshly isolated from blood. This revitalization process took place within the first 2 hr. and levelled out beyond that time. Repeated washing of these revitalized leucocytes did not change this new ability to reduce resazurin. It can be concluded that necessary cofactors were absorbed from plasma which allowed the leucocytes to oxidize substrates at a high rate or that the materials absorbed actually allowed new enzyme formation.

One often encounters milks from individual cows which reduce resazurin rapidly and yet contain only a few thousand bacteria and 200,000 leucocytes per milliliter. A possible explanation is that the leucocytes have lysed and released high concentrations of substrates which act as hydrogen donors for resazurin reduction. To test this possibility, fresh leucocytes, isolated from bovine blood, were suspended at a concentration of 5,000,000 per milliliter and disrupted by treatment for 30 min. in a 10-Kc. Raytheon sonic oscillator. Microscopic examination indicated that essentially 100% of the cells was broken by the treatment. A portion of the extract was heated at 85° C. for 15 min. to inactivate the enzymes and render the extract merely a source of substrates. Moreover, ribonuclease (RNase) or desoxyribonuclease (DNase) was added at the rate of 0.1 mg. per milliliter to both the heated and unheated extracts and the mixture incubated at 30° C. for 1 hr., in an effort to further degrade the larger components.

TABLE 5

Influence of disintegrated leucocytes on the rate of resazurin reduction in raw milk

	Reduction time (min.)
a. Milk	495
b. Milk + leucocytes	10
c. Milk + disintegrated leucocytes	155
d. Milk + heated disintegrated leucocytes	360
e. c after treatment with RNase or DNase	155
f. d after treatment with RNase or DNase	360

As would be expected, the disintegration of leucocytes by sonic oscillation resulted in the destruction of a large percentage of the dehydrogenase activity (Table 5). It is not possible to determine the contribution which the dehydrogenases of lysed leucocytes make towards the reduction of resazurin in raw milk. From the results with the heated extract, and with the unheated and heated extracts which were treated with RNase and DNase, one can conclude that the substrates liberated as a result of the disintegration of leucocytes have a minor influence on the rate of reduction of resazurin. Since milk has a plentiful supply of xanthine oxidase which will rapidly reduce resazurin on the addition of substrate, one can conclude that disintegrated leucocytes, even when treated with RNase and DNase, at the most supply only minute amounts of substrate which can be used by this very nonspecific enzyme.

From our work, it is evident that fresh bovine leucocytes have a strong ability to reduce resazurin when added to milk, and a concentration of 1,000,000 per milliliter will reduce resazurin to the 7/4 standard within 20 min. However, this activity is rapidly lost when the leucocytes are held in milk at 37° C. for 4 hr. This observation was confirmed by the fact that leucocytes isolated from milk had to be added at a concentration of 4,000,000 per milliliter to get significant increases in the rate of resazurin reduction. Furthermore, the removal of bacteria and leucocytes from raw milk by centrifugation had only a minor influence on the reduction time.

These data are in agreement with the conclusions of earlier workers (7), that leucocytes do not have a significant influence on the dye reduction tests. It was interesting to note that leucocytes isolated from milk rapidly regained strong reducing powers when incubated with bovine plasma for 2 hr. In addition to the fact that intact leucocytes, as they occur in milk, do not have strong reducing powers, it has been shown that disintegrated leucocytes do not supply substrates for the reducing systems of raw milk. It would appear, therefore, that the ability of the resazurin test to detect mastitic milk is based on the presence of some agent other than leucocytes or that, in these cases, there is sufficient serum in the milk to keep the leucocytes in a state of strong reducing activity.

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CRITICAL FEATURES OF GOOD DAIRY FEEDING EXPERIMENTS[1]

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SUMMARY

This paper is an analytical survey of the factors to be considered in designing dairy feeding experiments. The problems of choosing experimental subjects, treatments, and response measures, the desirability of estimating experimental errors and the concepts of experimental sensitivity and experimental efficiency are discussed in a general way. Matters of error control and experimental design are discussed in detail, with special reference to dairy experiments. To point up the logical basis of error control, attention is focused on the lactation curve and factors affecting its general trend and shape. Designs for continuous and change-over trials are discussed in terms of controlling the factors affecting the lactation curve. Carry-over effects in change-over trials and ways proposed to surmount them are considered. Some comments to guide researchers in choosing between continuous trials and between different types of change-over designs are made.

Features of good experiments.

Good dairy feeding experiments, as is true of all good experiments, are characterized primarily by

A. Proper choice of experimental subjects, of treatments to be imposed, and of response factors to be measured

B. Estimability of experimental error

C. Maximum possible experimental efficiency; i.e., satisfactory sensitivity with a minimum expenditure of resources or maximum possible sensitivity permitted by employable resources.

These features are listed in hierarchical order. Item A is prime. If the subjects selected, the treatments imposed, and the response factors measured do not suit the purpose of the experiment, it is not a good experiment, regardless of how well the remaining items are attained. In some sorts of experiments, too, sensitivity is affected by the treatments imposed. Item B must be placed above C, because if B is met, one can judge the reliability of experimental results, regardless of whether Item C is satisfied or not. Nevertheless, Item C must be taken as seriously as Items A and B in the planning of experiments. To answer some research questions, a definite sensitivity is required, and it is prudent to attain the desired sensitivity as economically as possible. When resources are limited, it is reasonable to use them as effectively as possible; otherwise, the fruitfulness of good planning with respect to Items A and B will be reduced.

Scope of presentation.

Without a specific research problem in mind, it is possible to discuss Item A only in a brief and general way. Also, only brief remarks will be made concern-

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ing Item B, since the principles involved are given in a number of well-known books on applied statistics.

Topics under Item C are divided into two groups. The first group consists of abstract matters that are important not only in dairy feeding work but in many fields of experimentation. Only general ideas are outlined in this paper. More detail, with example applications to dairy feeding work, is available (28).

The second group of topics under Item C encompasses matters specific to dairy feeding experiments. Primary among these is the matter of error control, and this topic will be given detailed attention. Discussion will be developed with the usual production measures (milk, fat, and FCM yields) and fat percentage assumed to be the response measures of major interest. Other measures will be mentioned occasionally, but requisite information about them is limited.

CHOICE OF SUBJECTS, TREATMENTS, AND RESPONSE MEASURES

Experimental subjects.

The subjects chosen should represent the population to which results are to apply. For example, first-calf heifers should be used if one wishes to study something particularly important for first-calf heifers. Sometimes representativeness is sacrificed by choosing uniform animals for the purpose of reducing experimental errors. As will be seen, proper design and analysis of most sorts of dairy feeding experiments lead to acceptable errors even when the cows are heterogeneous, and representativeness need not be sacrificed to obtain error control.

Treatments.

Decisions regarding the particular factors to be varied, to obtain the experimental treatments, depend on the purpose of an experiment. Care should be taken that choice of treatment factors is not influenced in harmful ways in the process of choosing a design or anticipating a method of statistical analysis. Sometimes patterns found in statistical textbooks are not adaptable to a problem, and it is advisable to seek the aid of a statistician.

In some experiments, treatments correspond to graded levels of one or more factors. Selection of levels is not entirely an arbitrary matter, because experimental sensitivity can be affected appreciably by choice of levels. Within the bounds delimited by biological and/or practical considerations, it is often possible to find a set of levels that are optimum, or nearly so, for the problem at hand. For example, if previous experience indicates that the relation of production rate to the level of a factor is acceptably closer to linear, and if the goal is to establish the intercept and slope of the relationship, only two levels of the factor should be used; namely, the lowest and highest possible within the permissible bounds, and observations should be evenly divided between the two levels. For more general considerations on the choice of levels, one may refer, for example, to Box (2), Box and Lucas (3), and Cochran and Cox (11). An attempt to choose levels suitable for a particular purpose in dairy experiments is seen in the work of Heady (22).

Response variables measured.

Some sorts of dairy feeding experiments might be more informative if measures in addition to the usual ones on production and body weight were made. For example, concentrations of certain constituents in milk (in addition to fat content) and in blood might be observed, as might digestibility. Such extra response items are, of course, sometimes made. Ordinarily, supplementary response variables should be measured only when there is a concrete hypothesis in mind; i.e., they should not be measured on the basis of a vague hope that they will be informative.

In some kinds of dairy feeding experiments, interest lies basically in the partition of energy between maintenance, body stores, and milk production. Although trite, it is nevertheless important to note that changes in body weight do not reflect changes in energy stores, except in a rough way. It seems proper to encourage more intense research on methods of measuring fat stores and fill in the intact animal [see, e.g., Reid (36) and Emery (12)].

ESTIMABILITY OF ERROR

Desirability of estimating experimental error.

Experienced researchers have a feel for the credibility of results, even though they do no formal statistical analyses. Expectations of error magnitudes for various types of experiments will be given in a subsequent section and these could be helpful to the inexperienced. One might argue, therefore, that there is no real need to estimate error in most experiments. Even for the experienced researcher, however, failure to estimate error in individual experiments leads to a risk, of unknown and sometimes large magnitude, that incorrect conclusions will be drawn. Size of experimental error varies not only among response measures, but also with basal conditions and with the set of treatments studied. Estimation of error permits control of the risk of making erroneous conclusions.

Publication of the estimates of error obtained is a good service. These estimates are helpful to readers who wish to examine results from a standpoint different from that of the author, and they add to the fund of information useful for experimental planning.

Comments on the estimability of error.

Most dairy feeding experiments are designed and conducted in such a way that an unbiased or essentially unbiased estimate of experimental error can be obtained upon proper analysis. Even so, there are in the literature experiments for which proper error is not estimable, except from general experience; some have appeared in the past few years. It seems reasonable to expect, however, that this fault will be less frequently found in the future.

There are published experiments in which proper error was estimable but the statistical analysis was incorrect or absent. In at least one instance, an attempt to fit the data from an experiment into a textbook analysis led the authors to the discovery, noted in their summary and conclusions, that cows differ in

production rate. Some other examples in which sense of pertinence seemed to be forgotten could be cited. Statistical analysis of dairy feeding experiments is becoming common. Researchers should decide on a method of analysis with care and, if there is any doubt about the appropriateness of a method, the aid of a statistician should be sought.

EXPERIMENTAL SENSITIVITY AND EFFICIENCY

Criteria of sensitivity.

Either one of two criteria of sensitivity is usually employed. For the first criterion, it is assumed that the goal of an experiment is to detect deviations of a given response parameter from some hypothetical value for the parameter. For the second, it is assumed that interest lies in estimating the value of a response parameter within specified limits of error. The sensitivity is measured in terms of the assurance (probability) that a given quantitative deviation from a hypothetical value for a parameter will be detected, or that the observed limits of error for an estimated parameter will be less than some specified amount. The two criteria are of somewhat different stringency, but both are dependent on the same factors. An instructive discussion of sensitivity is given by Cochran and Cox (11).

Factors affecting sensitivity.

The factors affecting sensitivity and the direction of effect are outlined in Table 1. Approaching the matter of sensitivity in a quantitative way leads to answers for questions such as:

(a) How many replications are needed to achieve a specified assurance?
(b) What is the assurance level for a specified number of replications?
(c) How large must a treatment difference be in order to have a specified assurance of detecting it with a specified number of replications?
(d) In experiments involving graded levels of treatment variables, what is the best choice of levels?

Examples are given by Lucas (28).

The experimental unit.

The experimental unit in most dairy feeding experiments is the individual cow but, in some sorts of experiments the unit may involve a group of cows; e.g., in grazing experiments, where milk per acre is of interest, the unit is the pasture plot and all cows on it. Grazing experiments will not be considered in this paper, although the principles discussed apply. In change-over trials, the unit is assumed, for practical purposes, to be the cow-period.

Experimental efficiency.

Experimental efficiency can be thought of as the sensitivity attained per experimental unit. It is desirable that the sensitivity per experimental unit be

TABLE 1

Factors affecting experimental sensitivity

Factors affecting sensitivity		Effect on assurance level of an increase in the factor
Detection criterion	Limits of error criterion	
Magnitude of deviation from hypothesis	Magnitude of tolerable limits of error	Increases
Magnitude of experimental error per experimental unit[a]		Decreases
Number of experimental units (replications) per treatment		Increases
Efficiency factor of the design for estimating a parameter of interest[b]		Increases
Number of degrees of freedom with which error is estimated[c]		Increases
Significance level	Confidence level	Decreases as the significance and the confidence levels are made more stringent

[a] The experimental unit is explained on page 196.

[b] This is the product of two quantities, namely, $E\gamma$, where E is the efficiency factor for contrasting two treatments and γ is a quantity dependent on the nature of the parameter of interest and, in designs for fitting response curves or surfaces, on the actual levels of the treatment variables used [Lucas (28)].

[c] The number of degrees of freedom for error is not particularly important, except in small experiments.

maximized, because the cost of experimentation increases with the number of experimental units. If sensitivity is specified, one will wish to minimize the number of units required, or, if the number of experimental units is fixed, one will wish to attain the maximum sensitivity possible.

The significance (or the confidence) level and the magnitude of deviation from hypothesis (or the tolerable limits of error) are ordinarily fixed by the research problem at hand. Except in small experiments, then, experimental efficiency is determined essentially by the efficiency factor for estimating the parameters of interest and by the unaccountable variation between experimental units (unit error). In practice, the efficiency factor and the unit error must be balanced against each other, because choosing a design which reduces the unit error may also result in a reduction of the efficiency factor. For efficiency factors of designs to be considered in this paper, see Lucas (28).

ERROR CONTROL

Prefatory remarks.

The coefficient of variation in production rate between cows in groups available for experimentation is ordinarily of the order of 25% and may be higher. If nothing could be done to account for this variation, impossibly large numbers of cows would have to be used to detect the relatively small deviations from hypothesis that are of practical concern or to render the limits of error desirably small. It is not unreasonable, for example, to desire to detect treatment differences in milk production as small as 2 to 4%.

To understand the control of factors causing variability in milk production rate, it is enlightening first to consider the lactation curve, the lactation-to-lactation trends, and the factors affecting them. Most dairy experiments are restricted to single lactations on a given cow, but some involve several lactations on each cow. Single-lactation studies will be discussed in detail and multi-lactation studies will be discussed to the extent that present knowledge permits.

The lactation curve.

Milk production rate usually increases for a time after calving. After attainment of peak production rate, a relatively slow decline exists until about mid-gestation. Thereafter, the decline usually accelerates [see, e.g., Gaines and Davidson (16)].

The initial rise in production rate is variable between cows, both in amount and duration. It usually is completed within a month after calving, but in high-producing animals it may take 6 wk. There is also considerable variation between cows regarding the behavior of the lactation curve during the latter half of gestation.

A number of investigators [Turner and coworkers (42), Brody and coworkers (8), Gaines and Davidson (16), Gaines (14, 15), and Gooch (17)] have found, upon studying hundreds of records, that the lactation curve, between peak production and mid-gestation, is well described on the average by the exponential law,

$$Y = Y_o P^t \qquad \text{(Eq. 1)}$$

in which

Y = production rate at time t
Y_o = production rate at $t = 0$; *i.e.*, at the initial observation
P = the persistency factor; where $0 < P < 1$

Gaines and Davidson (16) have also found (Eq. 1) to hold for fat-production rate and, presumably, (Eq. 1) would hold for the production rate of other major constituents of milk.

The smooth curve expressed by (Eq. 1) can be thought of as the idealized lactation curve during the interval from peak production to mid-gestation. In practice, deviations from smoothness of variable magnitude and duration occur. If, however, all of a group of cows are subjected to the same sequence of environmental changes, each of the group is at some stage of lactation past peak production, yet none has passed mid-gestation, the individual lactation curves show time changes that are remarkably similar regarding time of occurrence and, percentagewise, regarding amplitude.

This similarity of time changes is well illustrated by the correlations that exist between production levels during different portions of the lactation for cows having a common environment. Correlations between the first and the second 5-wk. periods following peak production ordinarily exceed .95, and those between the first and the fifth 5-wk. periods, .80. Clear evidence of these high correlations is seen in the extensive statistical studies of Maynard and Myers (29), and earlier workers were aware of them. The strong correlations support

the view that there is some underlying law about the lactation curve which is closely stated by (Eq. 1).

Proper exploitation of the underlying law leads to unit errors of 4 to 11% in the usual types of single-lactation studies. Most aspects of error control in single-lactation studies distill into problems of controlling the nontreatment variables which affect the sizes of the correlations.

There also exist correlations between successive lactation periods. These are not as strong as those between successive portions of the same lactation. Data given by Maynard and Myers (29) indicate that experimental errors will be twice as large if previous lactation is used as a basis for assessing expected experimental production than if the 2-wk. production just prior to experiment in the same lactation is used. Plum (35) observed a correlation of .58 between randomly selected pairs of lactations on the same individual. Gowen (18) found correlations ranging from almost .8 for immediately adjacent lactations to about .5 when the records correlated had several lactations intervening.

Advantage of the interlactation correlations should be taken in multilactation studies. It is harder, however, to control the factors that affect the correlations than it is in single-lactation studies.

Components of experimental error.

The factors responsible for imperfect correlations between various portions of the lactation curve in single-lactation studies seem well encompassed by the following:

(a) Measurement errors

(b) Between-cow variations in nontreatment environment

(c) Inherent between-cow variations in the persistency factor

(d) Differential responses of cows to secular shifts in environment.

In multilactation studies, Item (c) is replaced by:

(c-1) Inherent between-cow variations in the trends, from lactation to lactation, of the total production per lactation.

Measurement errors.

Errors in weighing the amount of milk produced, in sampling and in analyzing for the amount of fat or other constituents of milk, contribute negligibly to the total experimental error. Consider, for example, the contribution of milk-weighing errors to the variance of the determination of the average daily milk production of a cow milked thrice daily during a 30-day period.

The contribution is

$$\sigma_m^2 = \frac{n\sigma_w^2}{d} \qquad \text{(Eq. 2)}$$

where σ_w^2 = the variance of a single weighing

n = number of milkings per day

d = number of days in the period of interest

Suppose the scales can be read only to the closest .5 lb., certainly an outside figure. Under the assumption that the weighing errors are rectangularly distributed, $\sigma_w^2 = 2^2(.5)^2/12 = 1/12$. Substituting in (Eq. 2) yields

$$\sigma_m^2 = \frac{3(1)}{12(30)} = .0082 \text{ lb.}^2$$

The expected experimental error variance for a situation in which this weighing variance would apply is in the range 1.0 to 2.5 lb.^{2}.

For most response variables it appears that measurement errors are negligible compared to the uncontrollable biological variations. Even in the case of body weight changes, variations in fill and water content of the animal are probably more important than weighing errors.

Between-cow variations in nontreatment environment.

Because of the effects of environment, in both the long and short term, on the production rate of cows, the correlations between performances in different periods of lactation, or between different lactations, will be reduced if secular environmental changes are not the same for all cows. Only if the treatments are compared under the same conditions of nontreatment environment will minimum experimental errors be realizable. Good experimental designs satisfy this requirement for environmental comparability.

Feeding management. Ad libitum feeding of roughage, the periodic adjustment of individual roughage allowances to the body weight of each individual, and the periodic adjustment of individual concentrate allowances to the production level of each individual are sometimes proper experimental procedures. Such practices can reduce the correlations between productions in different portions of the same lactations, or between productions in different lactations, somewhat below the correlations that exist when equalized feeding is used [Lucas (23)]. Under the equalized method, feed allowances are held invariant through time or are varied by some rule which renders the time changes in feed allowances the same (preferably percentagewise) for all cows, regardless of the behavior of an individual cow. Note that the changes in allowances are rendered the same for all cows, not the feed allowances. The level of feeding for each cow is, prior to the experiment, adjusted to its own individual characteristics mainly on the basis of body weight, age, condition, production level, etc. If equalized feeding is compatible with the purpose of an experiment, using it will tend to minimize experimental error. The method easily can be adapted to experiments in which different feeding levels are being compared. However, where interest lies, for example, in the amount of roughage which will be consumed ad libitum, it is possible only to equalize on the concentrates. A similar situation exists in grazing experiments.

Inherent between-cow variations in persistency and differential response to nontreatment environment.

Because the correlations between production rates at different periods in the same lactation decrease as the interval between the periods increases, it is

evident that persistency varies between cows. Sanders (37) found the variability in persistency between lactations on the same cow to be less than the variability of persistency between cows.

High producers tend to decline in production more rapidly than do low producers, even on a percentage basis. Gaines and Davidson (16), Gaines (15), Sanders (38), and Gooch (17) found a portion of the between-cow variation in persistency to be associated with the peak production rate, the correlation being about −.5 in Gurnseys, Jerseys, and Holsteins. This portion of the between-cow variation in persistency is automatically eliminated from experimental error by taking initial production into account. The portion not eliminated may include variations not only in general trend but also in shape of the lactation curve. For example, a cow with a strong urge to produce might, because it can not eat enough, call on body stores for a time to support production and then, when stores become low, show an accelerated decline in production rate.

Even if all cows on an experiment are subject to the same secular changes in environment, thus permitting removal of most of the environmental effects from experimental error, one would expect a residual moiety of disturbances attributable to environment. Each cow responds somewhat differently to the environmental changes (cow-environment interaction), with a resulting influence on the between-cow variation in shape of the lactation curve. For example, some cows may be less sensitive to temperature extremes than are others.

The between-cow variations in general trend and shape of the lactation curve can be studied by subdividing lactation records into periods and examining the between-cow variations of the linear, the quadratic, the cubic, etc. components of the period-to-period trend in production rate. Results of such a study (24) are summarized in Table 2. For comparative purposes, results are expressed on a per-cow, per-period basis as coefficients of variation. The estimates were obtained from actual experiments of the change-over type, after removal of treatment effects. The experimental periods were 4 to 6 wk. long, and most of the cows were in the portion of the lactation period between peak production and mid-gestation. Initial production was not taken into account as efficiently as it might have been in the four-period groups, and this seems to explain the high variation of the linear component for that group.

The data in Table 2 illustrate two points which seem to hold in the general experience of the author. First, even after taking initial production level into account, the linear component of the time trend is more variable than the higher-

TABLE 2

Between-cow variations in components of the time trend in production rate

Number of groups of cows[a]	Number of periods	Average coefficients of variation		
		Linear component	Quadratic component	Cubic component
11	3	5.9	5.0	....
3	4	9.1	4.7	4.7

[a] Most of the groups contained 12 cows.

order (shape) components. Second, the quadratic and the higher-order components have about the same between-cow variability. This shape variability can be thought of as the base error for the prevailing experimental conditions. The figures for quadratic and cubic components in Table 2 are within the usual range but higher than average. In one type of design to be discussed, the base errors are realizable.

In multilactation studies, the between-cow variation of lactation-to-lactation trends of production determines experimental error. Although total production in a lactation is related to both peak production rate and persistency, peak production rate is by far the more important of the two [Gaines (14, 15) and Gooch (17)]. Particular attention should be given, therefore, to factors which cause between-cow variation of the lactation-to-lactation trends in peak production.

Age of cow. From the findings of Brody (5), Gaines (15), Gooch (17), Sanders (38), and Turner (41), one can conclude that, if pre-experimental production rate is taken into account, age of cow ordinarily can be disregarded in single-lactation studies. Only if there is an appreciable number of first-calf heifers, or of animals in their ninth or subsequent lactation, need special provision for age be made in the experimental design and the analysis.

Production per lactation and peak production increase in a nearly linear way from the first through the eighth lactation and thereafter, decline with age [Brody (6), Gaines (15), Gooch (17), and Maynard and Myers (29)]. In multilactation studies, then, provision for age needs to be made only if some of the cows will have entered their ninth lactation during the trial.

Genetic factors. Variations in genetic make-up cause some of the variation in persistency and in the lactation-to-lactation trends in total production. It does not seem possible to do much about these genetic effects, over and above that which is automatically done by taking advantage of the intra- and inter-lactation correlations. The studies of Bartlett (1), Gaines (15), Maynard and Myers (29), and Sanders (38), along with experiences of the author, provide no evidence that variations of persistency and lactation-to-lactation trend between breeds are of any significance compared to variations between cows within breeds. This does not mean that for some purposes it is not proper to take breed into account when designing experiments.

Summarizing commentary.

It is implied in the foregoing discussion that one should strive to use, in single-lactation studies, only cows which will be in the interval from peak production to mid-gestation. To have enough usable cows at the right time, this may require special planning regarding breeding dates, or flexibility in the purchase and sale of cows. It is also implied that if all of the cows needed for an experiment can not be housed at the same location or started on experiment at the same time, account of time and place must be taken in the design and analysis of experiments.

EXPERIMENTAL DESIGNS

In discussing experimental designs, particular attention will be given to the matter of controlling the unit error.

Types of designs.

For dairy feeding work, as for much other animal work, it is convenient to classify experimental designs into two categories:

(a) Designs appropriate for continuous trials

(b) Designs appropriate for change-over trials

Continuous trials are those in which an animal, once placed on an experimental treatment, remains on that treatment for the duration of the trial. Change-over trials are those in which an animal receives a time-sequence of two or more treatments during the course of the trial.

The method of taking advantage of the correlations between productions in different portions of the same lactation, or in different lactations, is ordinarily different in the two types of trials. Also, special provision to remove inherent time trends in behavior and the effects of secular trends in nontreatment environment must be made in designing change-over trials. The method of removing the main effects of items such as starting time and location from experimental error is essentially the same in the two types of trials.

A feature of experimental design ordinarily found in both types of trials—a feature necessary to take advantage of the correlations between production rates at different times—is the use of at least two periods[2] of observation on each cow. In continuous trials there are basically two periods of observation,[2] but in the change-over trials there may be several periods.

Designs for continuous trials.

The basic design. The two periods of observation in continuous trials will be termed the standardization period (S.P.) and the comparison period (C.P.). During the S.P. all cows to be used should be handled in a standard manner, and their performances measured. All rules of procedure fitting for the C.P. should be followed rigorously during the S.P. At the end of the S.P., the cows are allotted to experimental treatment. The basic design pattern for continuous trials is shown in Table 3.

[2] There are some sorts of continuous trials with only one period of observation, e.g., an experiment in which experimental treatment is to start at birth or weaning and is to continue through one or more lactation periods. In such, advantage can not be taken of the correlations.

TABLE 3

The basic design pattern for continuous trials

	Cows in:			
Experimental period	Group 1	Group 2	. .	Group p
Standardization	Tr. 0[a]	Tr. 0	. . .	Tr. 0
Comparison	Tr. 1	Tr. 2	. . .	Tr. p

[a] Tr. 0 = a standard treatment which may be one of the experimental treatments.

As Bartlett (1) points out, the two periods properly are considered to be experimental periods; hence, the use of the terms standardization and comparison instead of the common terms, preliminary and experimental. It seems desirable to employ the term, preliminary period, for the time prior to the S.P. during which cows are adapted to the base experimental conditions and individual feed allowances are adjusted to proper base levels [Lucas (23)].

Length of experimental periods. In single-lactation studies, the optimum length of S.P. is 2 to 3 wk. [Bartlett (1), Lucas, unpublished]. Longer or shorter times ordinarily will lead to slightly increased experimental errors. No work has been done on length of S.P. for multilactation studies. The S.P. might be a full lactation. It seems, however, that a good procedure would be to use the first 3 mo., say, of the first experimental lactation as the preliminary period, and the remainder of that lactation as the S.P.

Minimum length of the C.P. is determined by the purpose of an experiment. It might be 10 or 20 or more wk. in single-lactation studies, or consist of two or more total lactation periods in multilactation studies. The shortest C.P. compatible with the experimental purpose should be used, however, because experimental errors increase with length of C.P.

Use of S.P. performance for error control. It has been the common practice for a long time to utilize S.P. performance in allotment of cows to treatment groups by the procedure of balancing; i.e., by assigning the cows to treatment groups in such a way that the average S.P. performances of the several groups are as close to identical as possible. Attempts are sometimes made to render the ranges of S.P. performance about the same in the several groups.

Balancing has been criticized on the grounds that it can lead to bias, and, in its place, random allotments have been recommended. Studies made by the author lead to the view that balancing is acceptable in dairy experiments. In fact, if balancing can be done well, it seems preferable to random allotment in small experiments, because it guarantees a maximum possible efficiency factor for the experiment (28). The acceptability of balancing rests on the fact that the relation of S.P. performance to C.P. performance, if all cows in a group are subjected to the same conditions, is essentially linear.

Regardless of whether balancing or random allotment is used, S.P. performance must be taken into account in the analysis of the data. Several methods of doing this have been used to some extent, but the most efficient approach appears to be covariance analysis. Bartlett (1) has discussed covariance analysis for continuous trials with dairy cattle, and the requisite arithmetic is described in many statistical texts.

Control of factors affecting the correlations between S.P. and C.P. performances. It may happen that

(a) Calving dates and/or mid-gestation dates are such that some cows have to be started on experiment at different times than do others.

(b) To be able to obtain a sufficient number of observations, some of the cows must be housed at one location and some at other locations.

The time course of production, both inter- and intra-lactational, no doubt would vary between starting times and between locations, and provision for this should be made in design and analysis.

The principle of blocking, as symbolized in Table 4, can be invoked. Blocks can correspond to starting time, location, or to a combination of the two. It is desirable that the number of cows per treatment be uniform within a block, but the number can vary between blocks.

TABLE 4
The blocked design pattern for continuous trials

Block	Experimental period	Cows in: Group 1	Group 2	...	Group p
1	Standardization	Tr. 0	Tr. 0	...	Tr. 0
	Comparison	Tr. 1	Tr. 2	...	Tr. p
2	Standardization	Tr. 0	Tr. 0	...	Tr. 0
	Comparison	Tr. 1	Tr. 2	...	Tr. p
.	.	.	.	.	.
.	.	.	.	.	.
.	.	.	.	.	.
n	Standardization	Tr. 0	Tr. 0	...	Tr. 0
	Comparison	Tr. 1	Tr. 2	...	Tr. p

In the analysis, the variation associated with blocks is removed from the estimate of experimental error. Covariance on S.P. performance is employed as in the basic pattern. The effectiveness of the covariance adjustment is so great that production level ordinarily should not serve as a basis for forming blocks in continuous trials. If production-level by treatment interaction is expected to be noticeable, however, blocking on production level may be desirable.

Further ramifications of the design and analysis of continuous trials are pointed out by Lucas (27).

The experimental unit and expected unit errors. In practically all continuous trials, the experimental unit is the individual cow. Proper design and analysis of single-lactation continuous trials in which S.P. performance can be taken into account leads to unit errors in the ranges shown in Table 5. The figures apply for milk, fat, and FCM production and for fat percentage.

No direct estimates of errors for multilactation continuous trials are available. The interlactation correlations cited previously suggest that errors no lower than 15% would be expected, even with careful planning and conduct of experiment, and that errors as high as 25% could occur.

TABLE 5
Unit errors for continuous trials

Length of CP	Expected errors[a]
(wk.)	*(%)*
10	4– 8
20	7–11
30	11–15

[a] Coefficients of variation.

Unit errors with monozygotic twins. There have been many reports to the effect that experimental errors may be reduced greatly by using monozygotic twins. From uniformity data on lactating twins, Patchell (30) computed that one pair of twins is equivalent to 3.2 to 5.5 pairs of randomly selected animals when studying yields of milk and milk constituents, and to 23.3 random pairs for fat percentage. He notes that these figures may be optimistic for actual experimental work because of interaction of genetic make-up with environment. Patchell's figures are conservative compared to those of some earlier workers.

Hancock (21) reports a feeding experiment involving nine sets of twins. Two treatments were compared in a continuous trial with a 3-wk. S.P. and an 8-wk. C. P. For milk production, fat production, and fat percentage, he obtained unit errors of 6.2, 7.5, and 2.3%, respectively. Comparing with Table 5, some advantage for twins is indicated with milk-composition measures (such as fat percentage) but not for production measures. Other studies reported by Hancock (19, 20, 21), seem to warrant the conclusion that there is no advantage in employing monozygous twins to study feeding effects on milk production, except when the use of an S.P. is not compatible with the purpose of an experiment; e.g., when treatments are to be initiated prior to the first calving.

Designs for change-over trials.

Change-over trials have been popular in dairy cattle work for many years. They have intuitive appeal from the standpoints of error control and the number of observations per treatment that can be made with a given number of cows.

Questions have been raised regarding the validity of change-over trials. Two main points are involved; namely, that carry-over or residual effects of preceding treatments mask the effects of current treatment and that, even if carry-over effects do not exist, there possibly is a bias in estimating experimental error. Associated with the problem of carry-over effects and with some other considerations of minor nature, is the matter of optimum period length, particularly in single-lactation studies.

A considerable amount of work has been done on the matter of designs and methods of analysis intended to surmount the problem of carry-over effects. Bias in the estimate of error also has been investigated. The question of period length is a moot one. The author instinctively prefers periods of 4 wk. or more for most feeding trials wherein lactation is concerned.

Types of change-over trials. Change-over trials are basically of two types:

(a) Rotational (cyclical or round-robin) types

(b) Switch-back (reversal) types

and these are distinguished by the nature of the sequences of treatments employed. The basic rotational types are those in which the treatments in a sequence are all different. The switch-back types involve repetition of one or more treatments, but the repetitions do not occur contiguously.

In both types, the treatment sequences can not be the same for all cows. Two or more different sequences must be imposed, to eliminate from experimental

error the average inherent time trend in the performance of cows and the effects of secular changes in nontreatment environment.

In both types of trials, as in the continuous trials, preliminary and standardization periods are desirable. The S.P. serves the valuable purpose of permitting the cows finally to stabilize to the base experimental conditions. Although performance during the S.P. is not ordinarily utilized in the analysis of change-over trials, it should be used in the design of rotational type trials, in order to group cows most advantageously for exploiting the intra- and inter-lactational correlations in performance. In switch-back trials, S.P. performance can be completely disregarded in allotting cows to treatment sequences as well as in analysis.

Rotational designs. The most familiar designs for rotational trials are the Latin-squares. A pattern for three treatments is shown in Table 6. Ordinarily,

TALBE 6
A Latin-square change-over design

	Treatment sequence		
Comparison period	1	2	3
1	Tr. 1	Tr. 2	Tr. 3
2	Tr. 2	Tr. 3	Tr. 1
3	Tr. 3	Tr. 1	Tr. 2

several cows will be placed on each treatment sequence in small designs. Extension of the Latin-square pattern to any number of treatments is obvious, but there is usually a limitation on the number of comparison periods that can be used. In single-lactation studies, if periods are to be of reasonable length (4 to 6 wk.), the number of periods ordinarily must be limited to three, four, or five. When the number of treatments exceeds the possible number of periods, incomplete Latin-square patterns can be used (33). More extensive comments on this point are given by Lucas (27).

Switch-back designs. The basic pattern for switch-back trials involves two treatments in a three-period pattern with two sequences, as shown in Table 7. Again, several cows are placed on each sequence in small designs. The alternation scheme can be continued through four or more periods, but this appears to be of no advantage.

Switch-back patterns can be constructed for three or more treatments, still maintaining the three-period feature [Seath (39), Taylor and Armstrong (40), and Lucas (25)].

TABLE 7
The basic switch-back pattern

	Treatment sequence	
Comparison period	1	2
1	Tr. 1	Tr. 2
2	Tr. 2	Tr. 1
3	Tr. 1	Tr. 2

Provision for carry-over effects. Except for the devices of using comparison periods as long as possible, and of basing interpretations only on performance during the latter parts of the periods, little can be done about carry-over effects in switch-back trials. The same devices can be employed in trials of the rotational type, but more desirable procedures are available if certain assumptions regarding the nature of carry-over effects hold closely in practice.

Cochran (10) devised Latin-square designs and corresponding analyses which permit estimation of and correction for carry-over effects under the assumption that the carry-over effect of a treatment is independent of the treatment with which it occurs. This assumption has characterized essentially all of the work on carry-over effects to date and seems adequate for many situations. There are instances, however, in which the assumption does not apply, and work on the matter is needed.

A distinctive feature (the symmetry feature) of Cochran's designs is that each treatment is immediately preceded by each other treatment the same number of times. For three treatments, six sequences (two squares) are required, as shown in Table 8.

TABLE 8

A Latin-square change-over design balanced for carry-over effects

Comparison period	Treatment sequence					
	1	2	3	4	5	6
1	Tr. 1	Tr. 2	Tr. 3	Tr. 1	Tr. 2	Tr. 3
2	Tr. 2	Tr. 3	Tr. 1	Tr. 3	Tr. 1	Tr. 2
3	Tr. 3	Tr. 1	Tr. 2	Tr. 2	Tr. 3	Tr. 1

Under the assumption that carry-over effects persist only one period, Williams (43) proved that symmetry can always be attained with one Latin-square when the number of treatments is even, and with two squares when the number of treatments is odd. Williams (44) also found sets of Latin-squares which are symmetrical for carry-over effects persisting for two periods. To permit the number of periods to be less than the number of treatments, Patterson (32, 33) developed sets of incomplete Latin-squares that are balanced for carry-over effects lasting one period.

The basic patterns of complete and incomplete Latin-squares balanced for carry-over effects can be extended by continuing the final treatments into an additional period [Lucas (26) and Patterson and Lucas (34)]. This provides patterns in which each treatment is immediately preceded the same number of times by every treatment including itself. The extra-period designs are particularly good for estimating carry-over effects that persist one period. Federer (13) presents some designs in which the doubled period is not always the final one.

Components of error in change-over trials. Because each cow receives two or more treatments, and because the several treatments appear in each comparison period in change-over trials, it is possible to find ways of contrasting treatments, such that the error of the contrasts contains none of the between-cow and between-period variations in general production level. Remaining in error, however, are

the between-cow variations in time trend (period by cow interaction). Appropriate methods of analysis are described in the references cited in this section and the immediately preceding sections dealing with rotational designs, switch-back designs, and carry-over effects. They are considered also in texts such as Cochran and Cox (11) and Federer (13).

In trials of the rotational type, the between-cow variations in all components of the time trend (linear, quadratic, cubic, etc.) contribute to experimental error. The usual method of analysis does not, in general, give the several components proper relative weight, and a bias in estimating error can be introduced [Patterson (31), Lucas (24), and Patterson and Lucas (34)]. A correct analysis is laborious and hardly practical and, often, the bias is unimportant. If the bias is thought to be important, the usual analysis can still be used, with an approximate correction suggested by the authors just cited.

In switch-back trials, proper analysis [Brandt (4) and Lucas (24)] eliminates one or more components of the time trend from error. For example, in the three-period design, the linear component is eliminated, and in the four-period extension, linear and quadratic components are eliminated. Error in switch-back trials thus attains the base level.

Error control in change-over trials. As indicated previously, some of the between-cow variation in time trend is related to production level. Experience of the author indicates that the linear component of the trend provides most of the portion related to production level; i.e., the quadratic and higher-order components are related to production level in a negligible way, even when the range of production level of the cows is wide. Thus, production level must be taken into account in trials of the rotational type, but can be neglected in the switch-back type.

All components of the between-cow variations in time trend can be increased seriously if the sequence of nontreatment environmental conditions varies between cows. Thus, items such as starting time and location must be taken into account to realize minimum errors in both types of change-over trials, just as was the case for continuous trials. To avoid complications of analysis in rotational change-over trials, the S.P. performance is best taken into account by blocking procedures, instead of by covariance.

Blocking in change-over trials. The treatment sequences forming the basic pattern of a change-over design usually fall naturally into one or more, say g, groups (e.g., the two squares in Table 8). If n cows are placed on each sequence, it can be considered that the total number of blocks in the experimental design is gn. Effects of factors that can be made to coincide with blocks do not appear in experimental error and can be eliminated from the estimate of error by proper analysis.

Blocks can be made to correspond to starting times and/or locations. This is an effective error-reducing procedure in both types of change-over trials. If errors are to be minimized in the rotational types of trials, the cows within a given block should be as uniform as possible as regards S.P. performance.

Blocking and associated features of statistical analysis are discussed by Cochran (10) and Lucas (26) for trials of the rotational type, and by Lucas (25) for switch-back designs.

The experimental unit and unit errors. In change-over trials, as in continuous trials, the experimental unit strictly is the individual cow. The practical method of analysis for trials of the rotational type is based on the assumption, however, that the experimental unit is the cow-period (one comparison period on one cow). The possible bias in estimate of error is associated with this erroneous assumption. For experimental planning, it is satisfactory to consider the experimental unit to be the cow-period.

Analysis of switch-back trials is done under the correct assumption that the unit is the cow. Nevertheless, it is convenient, for comparative purposes and for experimental planning, to consider the cow-period to be the unit in switch-back designs.

In single-lactation studies of the rotational type, unit errors (per cow, per period) average between 5 and 6%, with a usual range of 1.5 to 9.5% [Burt (9), Lucas (26), and Taylor and Armstrong (40)]. These figures apply for fat percentage and for milk, fat, and FCM productions. They are affected but slightly by period length if periods are from 3 to 6 wk. long.

In single-lactation studies of the switch-back type the unit errors for production measures average between 2 and 3%, with a range of 1 to 7% [Lucas (26) and Taylor and Armstrong (40)]. In line with Table 3, the author has not found the unit errors in the three-period switch-back design to be much different from those for the four-period extension.

No work on errors for multilactation change-over trials has been done. Nevertheless, judging from the interlactation correlations given, it appears that the errors would be no less than 10% in switch-back types and as high as 20% in rotational types.

Choice of design.

Factors to consider in deciding on the type of design to use have been listed by Lucas (26), and the relative merits of the basic vs. the extra-period rotational type change-over designs have been examined rather carefully by Patterson and Lucas (34). However, until more work, both theoretical and experimental, is done only rough recommendations can be made on some points regarding choice of design.

From a consideration of the unit errors and of the efficiency factors [Lucas (28)], it seems clear enough that the three-period switch-back designs are the best to use if carry-over effects safely can be assumed to be negligible. If carry-over effects are not large, or do not persist very long, the switch-back designs also may be preferable if one is interested simply in detecting treatment effects and not in estimating the magnitude of treatment effects to be expected in practice.

If one anticipates carry-over effects that are large but persisting only one period, the rotational types of change-over trials may be the proper ones to use, particularly if detection of treatment effects instead of estimation is the main goal. In this case, the basic designs seem preferable to the extra-period designs.

If large carry-over effects seem likely, and interest lies in estimating permanent (direct plus carry-over) effects for practice, a choice must be made between the extra-period rotational type change-over trials and continuous trials. The extra-period change-over designs seem satisfactory for many of the ordinary feeding experiments. Actually, the matter depends on how well the assumptions about the nature of the carry-over effects that are made in design and analyis fit the real situation.

Finally, it seems clear that continuous trials should be used if the treatments involve variations of nutritional factors which might be stored in large amounts relative to weekly, monthly, or yearly need (e.g., the fat-soluble vitamins and certain minerals), and if interest lies in estimating quantitatively the long-run effects.

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EFFECT OF MILKING INTERVALS ON THE RATE OF MILK AND FAT SECRETION

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SUMMARY

Fifteen high-producing Holstein and Brown Swiss cows in early lactation were milked at 4-, 8-, 12-, 16-, and 20-hr. intervals. Ten I.U. of oxytocin were injected at the beginning and end of each interval to obtain residual milk. Each interval was repeated three times and each series of intervals was separated by a 12-hr. interval. The rates of milk, fat, and solids-not-fat secretion were linear for intervals up to and including 12 hr. Significant reductions in rates of secretion of these components were found at 16- and 20-hr. intervals between milkings as compared to the 4-, 8-, and 12-hr. intervals.

The butterfat percentages for the different intervals were nearly the same, except for the 4-hr. interval, where an unexplainable increase took place. The solids-not-fat content was the same for all intervals. Preceding long intervals caused a depression of milk secretion during the 12-hr. intervals, but caused a marked increase in butterfat percentages.

The rate of milk and fat secretion during various time intervals is an important factor in obtaining maximum production from dairy cows. The theory that increased udder pressure has a depressing effect on the rate of milk and fat production is used to explain the increased production resulting from milking cows more frequently. It is also the basis for advocating the milking of cows at equal intervals. Controlled experiments (1, 5, 7, 9, 14) have shown that the rate of milk secretion is depressed by milking intervals over 12 hr., but that the butterfat percentage is increased with longer intervals. This work was questioned when Elliott and Brumby (4) and Turner (20) reported no significant depressions in the rate of milk secretion for intervals up to 20 hr. Dodd, Gibbons, and Elliott (3) also reported similar rates of secretion for milk, fat, and solids-not-fat for intervals up to and including 16 hr. when oxytocin was used to remove residual milk. Other experiments (8, 11, 13, 15, 22), comparing intervals of 12 and 12, 11 and 13, 10 and 14, and 8 and 16 hr. for longer periods of time, have shown very small differences in milk and fat production. All differences between intervals were less than 3.5%; however, no high-producing cows were used in these studies.

Long intervals preceding regular intervals have a depressing effect on the rate of milk secretion (1, 3, 4) and cause an increase in fat percentage (1, 4). Bailey, Clough, and Dodd (1) present evidence indicating that the milk yield is influenced by one preceding interval and the fat yield by two of three preceding intervals. This is also supported by the fact that the rate of milk secretion is reduced 15% when a 24-hr. interval is imposed in a regular twice-daily milking schedule (21); whereas, milking first- and second-lactation cows once daily for longer periods of time reduced milk production 50 and 40%, respectively, compared to twice-daily milking (2).

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The objectives of this experiment were to determine the effect of varying time intervals on the rate of milk and fat secretion of high-producing cows in early lactation, and to determine the effect of length of preceding intervals on the rate of milk and fat secretion.

EXPERIMENTAL PROCEDURE

Fifteen cows, 12 Holstein and three Brown Swiss, in early lactation were stratified according to a week's previous production and assigned to trios by placing one each at random from the high-, medium-, and low-producing groups in each trio. The cows were fresh an average of 47 days, with a range of from 21 to 71 days. The average week's production prior to the experiment was 64.4 lb., with a range of from 50.2 to 80.8 lb.

A 5×5 Latin-square was used, with milking intervals of 4, 8, 12, 16, and 20 hr. as the treatments. Each milking interval was repeated three times in succession and was followed by a 12-hr. interval. The experiment was analyzed by the procedure outlined by Snedecor (19) and treatment differences determined using Duncan's Multiple Range Test as outlined by Federer (6). The 12-hr. intervals also were analyzed, to determine the effect of preceding intervals.

Residual milk was obtained at each milking by injecting 10 I.U. oxytocin after the regular milking. Several days before the experiment began, a small polyethylene tube was inserted into the subcutaneous abdominal vein about 8 in. and held in place by branding-cement and adhesive tape. Oxytocin was injected into this tube after the normal milk was obtained. In this way, the milking machine was not removed from the cow, and one man did the milking. A 0.5% sodium citrate, sterile saline solution was used as the carrier for oxytocin. The method of injecting oxytocin has been reported by Dodd, Gibbons, and Elliott (3) and by Elliott and Dodd (5).

The cows were fed twice daily and according to the previous week's production. The time of milking-machine application was noted for each cow. The combined weight of regular and residual milk was recorded and a representative sample taken for butterfat and total solids determinations. The butterfat was analyzed by the Babcock method and the total solids were determined using a Quevenne lactometer for specific gravity and the butterfat test. The per cent total solids was calculated by using the formula reported by Sharp and Hart (16).

A serious problem arose from the effect of the subcutaneous abdominal tubes after they were removed. Ten of the 15 cows developed severe cases of phlebitis and infections around the subcutaneous abdominal veins. These infections developed within a few days to from 2 to 3 wk. after the removal of the tubes.

RESULTS AND DISCUSSION

Milk production. The results of the effect of the intervals on the rate of milk secretion are given in Table 1. It can be seen that the rate of secretion is the same through 12 hr., but a reduction occurs at 16 hr. and a further reduction at 20 hr. The rate at 16 hr. was significantly lower than the rates at 4, 8, and

TABLE 1

Pounds of milk secreted per hour by cows milked at various intervals with the aid of oxytocin

	Intervals (hr.)				
Milkings	4	8	12	16	20
First	2.72	2.74	2.76	2.65	2.49
Second	2.75	2.72	2.71	2.59	2.37
Third	2.72	2.81	2.79	2.52	2.41
Av.	2.73	2.76	2.75	2.59	2.43
Succeeding 12-hr. interval	2.77	2.74	2.72	2.69	2.51

12 hr. ($P<0.05$), and the 20-hr. secretion rate was significantly less than the rates during the other intervals ($P<.01$). There were no significant differences in the secretion rates in the order of milkings, but the secretion rates decreased during the second and third 16- and 20-hr. intervals. The rate of secretion during the succeeding 12-hr. interval preceded by 20-hr. intervals was significantly lower than the rest ($P<.01$). This indicates that previous long intervals have a depressing effect on the rate of milk secretion. These results are in general agreement with previous reports (1, 5, 7, 9, 14), indicating that the rate of secretion is linear for 12 hr. followed by a depression with increasing intervals. This is not in agreement with the work of others (3, 4, 20) who reported similar secretion rates for 16- and 20-hr. intervals.

The reductions in rate of milk secretion between the first 16- and 20-hr. milkings, compared to the first 12-hr. milking, are 3.3 and 9.1%, respectively. If the residual milk is considered to account for 20% of the total milk in the udder, the 16-hr. and 20-hr. intervals when residual milk is removed are equivalent to 12.8- and 16-hr. intervals, respectively, when no residual milk is obtained. If the decline in milk secretion rate is linear from 12 to 16 hr., the expected rate of secretion for a 12-hr. normal interval would be 2.68 lb. per hour. The expected rate of milk secretion in 8- and 16-hr. daily intervals, using first-milking figures, would be

$$\frac{8 \times 2.74 + 16 \times 2.49}{24} = 2.57.$$

In comparison to the rate of secretion during the calculated 12-hr. normal interval, this is a reduction of 4.1%. This figure is in close agreement with actual results obtained from testing these two intervals over a period of time (8). Using these same assumptions, the average rate of secretion expected in 10- and 14-hr. normal intervals would be 2.66, a reduction of less than 1% from the calculated 12-hr. normal secretion rate. Work is now under way testing 12- and 12-, 10- and 14-, and 8- and 16-hr. intervals for complete lactations.

The cows were divided into three groups according to production during the 11-day experimental period. The results are shown in Table 2. The highest producing group in this experiment was affected less, on a percentage basis, by long intervals than were the other two groups. This is supported by the work of Korkman (10), who showed that within the same stages of lactation the absolute increase in intramammary pressure is the same for high- and low-yielding cows and, consequently, the increase in pressure per unit of secreted

TABLE 2
Milk secretion rates of production groups and reduction in secretion rates from the first three intervals

Groups	No. cows	Av. prod. lb. milk	Milk secretion rates (lb/hr) Intervals (hr.) 4	8	12	16	20	Per cent reduction in secretion rate as compared to first 3 intervals Intervals (hr.) 16	20
High	4	71.2	3.02	2.97	3.11	2.99	2.84	1.6	6.6
Medium	5	63.8	2.72	2.84	2.79	2.62	2.48	5.8	10.8
Low	6	56.2	2.50	2.54	2.48	2.29	2.11	8.8	15.9

milk is smaller in the high-yielding cows. Turner (20) states that high-yielding cows are not necessarily the most susceptible to long intervals. These results do not agree with those of Lakshmanan *et al.* (12), who found that high-producing cows in early lactation increased production more than did moderate producers when milked at 2-hr. intervals with the aid of oxytocin. One of the limitations of the work of Lakshmanan *et al.* (12) is that all the 12, 2-hr. periods for which an increase was noted occurred in one 24-hr. period.

The present paper does not compare intervals at different stages of lactation but, in most reports, long intervals have had about the same effect in early lactation as in late lactation. Dodd, Gibbons, and Elliott (3) found the decline in rate of secretion less after 16 hr. in late lactation than in early lactation. Turner (21) showed the depression in milk secretion rate in a 14-hr. interval, as compared to a 10-hr. interval, to be 0.6% in early lactation and 6.3% in late lactation, but neither figure was statistically significant. Turner (20) also found that the net capacity of the udder declined during lactation in proportion to the concurrent decline in daily yield, and concluded that any given long interval between milkings reduced yield in late lactation to almost the same degree as in early lactation.

Butterfat per cent and yield. The butterfat percentages for the milking intervals and succeeding 12-hr. intervals are given in Table 3. Each interval

TABLE 3
Butterfat composition of milk and secretion rate as affected by milking intervals

Milkings	Interval (hr.) 4	8	12	16	20
	Butterfat per cent				
First	4.84	4.11	3.71	3.97	4.01
Second	4.09	3.95	4.21	4.03	4.25
Third	4.16	4.06	3.89	3.71	3.76
Av.	4.36	4.04	3.94	3.90	4.01
Succeeding 12-hr. interval	3.59	3.98	3.85	4.14	4.36
	Butterfat secretion rate (lb/hr)				
First	.13	.11	.10	.10	.10
Second	.11	.11	.12	.10	.10
Third	.11	.12	.11	.09	.09
Av.	.12	.11	.11	.10	.10
Succeeding 12-hr. interval	.10	.11	.11	.11	.11

figure is an average of 15 determinations. The average butterfat percentage for the 4-hr. interval is significantly greater than the four other intervals ($P<.05$). Most of this difference occurred in the first milking. It can be seen that the butterfat percentages for the other intervals are nearly the same.

Ragsdale, Turner, and Brody (14) and Johansson (9) reported decreased butterfat percentages with increasing intervals, but the residual milk was not considered. The present work is in agreement with Garrison and Turner (7), who found no differences in milk composition in intervals up to 18 hr., but it differs from the work of Bailey, Clough, and Dodd (1) and Turner (21), who found slight increases in butterfat percentages with increasing intervals. The significant increase in the butterfat per cent for the 4-hr. interval is contrary to all literature reviewed and no explanation for this is apparent. Shaw (17), Smith (18), and Lakshmanan *et al.* (12) all showed marked reductions in butterfat percentages when cows were milked at 2-hr. intervals with the aid of oxytocin.

The butterfat percentages for the succeeding 12-hr. intervals increase with the length of the preceding intervals. The butterfat percentage for the succeeding 12-hr. interval preceded by the 20-hr. intervals is significantly greater than those preceded by the 4-, 8-, and 12-hr. intervals ($P<.05$). The butterfat percentage during the one preceded by the 16-hr. intervals is significantly greater than the one preceded by the 4-hr. intervals ($P<0.05$). These results are in agreement with those of Bailey, Clough, and Dodd (1), who found the fat yield to be influenced by the length of two or three preceding intervals.

The rates of fat secretion also are given in Table 3. The average rate of fat secretion during the 4-hr. interval is statistically greater than the 12-, 16-, and 20-hr. intervals ($P<.05$), and the rates during the 8- and 12-hr. intervals are statistically greater than the rate during the 20-hr. interval ($P<.05$). The higher rate of fat secretion during the 4-hr. interval is due primarily to the high butterfat test. The other differences are due to the decreased rates of milk production. The rates of fat secretion during the succeeding 12-hr. intervals are not statistically different, reflecting that the lowered rates of milk secretion are offset by the higher butterfat percentages.

TABLE 4

Solids-not-fat composition of milk and secretion rate as affected by milking intervals

Milkings	Interval (hr.)				
	4	8	12	16	20
	Per cent solids-not-fat				
First	9.20	9.17	9.31	9.36	9.31
Second	9.22	9.28	9.29	9.32	9.25
Third	9.35	9.32	9.44	9.43	9.40
Av.	9.25	9.26	9.35	9.37	9.32
Succeeding 12-hr. interval	9.33	9.48	9.43	9.46	9.42
	Solids-not-fat secretion rate (lb/hr)				
First	0.25	0.25	0.26	0.25	0.23
Second	.25	.25	.25	.24	.22
Third	.25	.26	.26	.24	.23
Av.	.25	.26	.26	.24	.23
Succeeding 12-hr. interval	.26	.26	.26	.26	.24

The solids-not-fat composition and secretion rates are given in Table 4. The solids-not-fat composition is nearly identical for all intervals; therefore, the solids-not-fat secretion rates are in direct proportion to the milk secretion rates. These results point out that, except for the butterfat percentage, the composition of milk is not markedly changed by milking intervals. These results are in agreement with those of Garrison and Turner (7).

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THE NET ENERGY OF BLACKSTRAP MOLASSES FOR LACTATING DAIRY COWS[1]

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SUMMARY

The net energy of cane or blackstrap molasses for lactation has been determined when added to a basal ration of pineapple bran, soybean meal, dicalcium phosphate, and trace-mineralized salt. When fed at a level of 10 and 30% of the total ration, the net energy of the molasses was 68.1 and 23.1 meal. per 100 lb., respectively, compared to 54.2 meal. for the basal ration. A comparison of the net energy values with total digestible nutrients and digestible energy indicated that the high loss of energy when molasses was increased from 10 to 30% in the ration was not due to increased fecal loss.

The addition of 30% molasses caused a significant decrease in percentage of butterfat and solids-not-fat in the milk, resulting in a lowering of the energy value per unit of milk produced. Milk from the cows fed 30% molasses also tended to score lower than the other milks in flavor tests. The lowered flavor score appeared to be associated with an increased rancidity. Ten per cent molasses produced none of the undesirable effects exhibited by the 30% molasses.

The caloric value of the milk solids was found to be very closely related to the fat content of the milk ($r = 0.98$). The relationship was described by the equation $Y = 4.516 + 0.321X$, where Y is the kilocalories of energy per gram of milk solids and X is the per cent butterfat in the fluid milk. The error of the regression coefficient was 0.043.

Cane or blackstrap molasses is often one of the most economical sources of carbohydrates for animal feeding. A search of the literature reveals numerous feeding experiments in which blackstrap molasses has been used as an ingredient in dairy cattle rations. Scott (13) extensively reviewed the literature available to 1953. Despite the length of time molasses has been used as a feed for dairy cattle, little information is available on its net energy (NE) value for lactation. The reason for this lack of information is that NE, as classically determined, requires the use of a respiration calorimeter and accumulation of information is slow and expensive.

Experiments have indicated that there is an optimum level of feeding molasses and that higher levels result in lowered production. There seems to be little agreement, however, as to the level at which optimum response is obtained. Scott (13) concludes that under unfavorable conditions, blackstrap molasses is about 75% as valuable as corn or other grains, but indicates that the average value is close to 85% of that of corn. If the replacement value differs with varying levels, it seems important to establish that level at which maximum feeding value can be achieved.

Because of the abundant supply of molasses, its importance in the feed industry, and the lack of information on its NE value when fed at different

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[1] Approved by the Director of the Hawaii Agricultural Experiment Station as Technical Paper No. 459.

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levels to lactating dairy cows, studies were designed to provide information on this subject.

DEVELOPMENT OF THE METHOD

The NE content of a feed can be defined as the energy expended for maintenance (M), plus that appearing in products such as milk, meat, work, etc. *(P)*. Simply expressed:

$$NE = M + P \tag{1}$$

The estimation of M becomes unnecessary if the feed or ration is fed at two levels, both above maintenance and the energy yield due to the feed increment (NE_1) determined. NE_1 is equal to the NE in the feed at the higher level (NE_2), minus that at the lower level (NE_1) of feeding and can be expressed:

$$NE_1 = NE_2 - NE_1 \tag{2}$$

By substitution:

$$NE_1 = (M_2 + P_2) - (M_1 + P_1) \tag{3}$$

$$= M_2 - M_1 + P_2 - P_1 \tag{4}$$

If the experiment is properly designed so that each cow receives each treatment, such as in a Latin-square design, M_1 and M_2 are equal and the equation becomes:

$$NE_1 = P_2 - P_1 \tag{5}$$

In lactation trials, it is difficult to hold the body weight of the experimental animals constant. In this case, P must include the energy in the body weight change as well as that in the milk. Inclusion of the energy in the body weight change is not inconsistent with the definition of P.

It is obvious from these considerations that the NE of any feed or combination of feeds can be determined if P can be measured with a reasonable degree of accuracy. The energy in the milk can be readily and accurately measured by determination of the heat of combustion of the milk solids and the percentage of total solids in the fluid milk. The energy equivalent of the weight change can be estimated from data in the literature. Brody (3) indicates that each pound of weight change in lactating dairy cows is equivalent to 2.1 lb. of total digestible nutrients (TDN). Assuming that there are 1,616 kcal. of metabolizable energy per pound of TDN [Forbes and Kriss (5)] and that metabolizable energy in a good mixed ration is used for weight gain at an efficiency of 57.5% [Forbes *et al.* (6)], it can be determined that 1 lb. of weight change in lactating cows is equivalent to 1.952 mcal. of NE. One would expect that any error in estimation of the energy equivalent of the weight change would have only a small effect upon the estimation of the total energy yield, since the energy in the milk normally greatly exceeds that in the weight change.

Based upon the considerations presented above, it appears feasible to determine the net energy in experiments with lactating dairy cows according to the concepts developed in deriving Equation (5). This paper reports results of studies designed to determine the NE value of molasses by such a method.

EXPERIMENTAL PROCEDURES

Eight Holstein cows just past their peak in lactation were divided into two groups of four cows each according to date of calving and assigned to two blocks of the following extra-period Latin-square design [Lucas (9)].

Block I

Period No.	Cow No.			
	330	411	419	448
1	Restricted basal	Basal	Basal plus 30% molasses	Basal plus 10% molasses
2	Basal plus 10% molasses	Basal plus 30% molasses	Restricted basal	Basal
3	Basal plus 30% molasses	Basal plus 10% molasses	Basal	Restricted basal
4	Basal	Restricted basal	Basal plus 10% molasses	Basal plus 30% molasses
5	Repeat of Period 4			

Block II

Period No.	Cow No.			
	316	323	413	422
1	Basal	Restricted basal	Basal plus 30% molasses	Basal plus 10% molasses
2	Basal plus 10% molasses	Basal	Restricted basal	Basal plus 30% molasses
3	Restricted basal	Basal plus 30% molasses	Basal plus 10% molasses	Basal
4	Basal plus 30% molasses	Basal plus 10% molasses	Basal	Restricted basal
5	Repeat of Period 4			

This design was chosen since it was felt there may be carry-over effects of some of the treatments into the next period, and such a design provides a method of assessing the magnitude and significance of any carry-over effects. Cows within each block were assigned at random to ration sequences and were started on experiment at the same time.

The basal ration consisted of 66% pineapple bran and and 34% soybean meal. A supplementary source of calcium and phosphorus plus trace minerals was supplied by allowing free access to a 50 : 50 mixture of trace-mineralized salt and dicalcium phosphate. The average daily consumption of this mixture was approximately 280 g. per cow. The protein content was such that on the restricted level of basal, the protein furnished was sufficient for full production. Energy thus was the only limitation. Analyses in this laboratory indicate that pineapple bran contains sufficient carotene to meet the needs of the dairy cow when fed at these levels.

Prior to the initiation of the experimental period, the cows in each block were placed on a 2-wk. preliminary period during which the basal ration was fed and adjustments made in feed allowances according to production level and body condition. This was followed by a 2-wk. standardization period during which the cows were held on a constant intake of basal ration and production allowed to become adjusted to changes made during the preliminary period. The production during the last week of the standardization period was the base level used to determine the lactation allowance for the experimental periods. The total feed allowance for each cow was determined by calculating the amount of basal required for maintenance, this amount being held constant throughout the entire study. The lactation allowance was held constant during each 5-wk. experimental period, but at the beginning of each succeeding period was reduced at the rate of 1.75% per week to allow for normal decline. The amount of feed required for lactation was added to the maintenance requirement to equal the total allowance. In the case of the restricted basal, only half the lactation allowance was added to the maintenance requirement. The two rations containing molasses were fed at the same level as the full allowance of basal. Some feed refusals on the 30% molasses ration, however, resulted in a slightly lowered consumption. The upper levels of the Morrison feeding standards were used in determining allowances.

Each experimental period was of 5 wk. duration, with the first week being used as a transition period. Only the data from the last 4 wk. of each period were used in the analysis. Prior to the initiation of each period, the entire quantity of feed for each cow was weighed and packaged for twice-a-day feeding, at which time samples were taken for analysis.

The TDN, digestible crude protein (DCP), and digestible energy (DE) were determined on all rations in conventional digestion trials with dairy steers weighing approximately 1,000 lb. Six trials were conducted on the basal and the basal plus 10% molasses and five on the basal plus 30% molasses. TDN was calculated from digestible organic matter (7).

Body weight measurements were made on three successive days at the beginning and end of each period immediately after milking and prior to feeding. Cows were milked twice daily and were fed twice daily after milking.

Milk samples were collected the last six milkings of each week, composited for each cow and analyzed for total solids, fat, and energy. Total solids were determined by evaporating 5 g. of milk in flat-bottomed moisture dishes over steam for approximately 20 min., followed by drying under vacuum at 98–100° C. for 2 hr. Fat was determined by the Babcock test. Energy determinations were made by freeze-drying a 30-ml. sample of milk and combusting approximately 1 g. of pelleted solids in an oxygen bomb. Gross energy of feeds was determined on approximately 1-g. pellets. The pelleting of feed samples was facilitated by an addition of approximately 1 ml. of water to 10 g. of sample. Dry matter on the lyophilized milk and the feed samples was determined by drying under vacuum at 98–100° C. for 2 and 5 hr., respectively.

TABLE 1

Composition of the molasses[a]

	Mean	Range
Moisture *(%)*	22.4	21.8–23.2
Ash *(%)*	10.5	9.8–11.9
Degrees Brix	82.0	81.1–83.0
Total sugars *(%)*	55.5	52.6–57.6
Crude protein *(%)*	1.9	1.5– 2.8

[a] Molasses analysis made by Hawaiian Sugar Planters' Association Experiment Station, Honolulu.

The last two milkings of each week were refrigerated immediately upon collection by use of a portable ice chest. They were then held overnight under refrigeration and tested for rancidity and flavor score.

RESULTS AND DISCUSSION

The composition of the molasses is presented in Table 1 and the complete rations in Table 2.

There were no carry-over effects in any of the criteria of response determined in these studies. The direct effects were, therefore, used in evaluation of the response to treatments.

The data presented in Table 3 show that the ration containing 30% molasses produced significantly less milk energy and total energy than either the basal or the basal plus 10% molasses. In terms of either milk energy alone or total energy, the addition of 30% molasses was equivalent to reducing the lactation allowance by half, since there was no difference between the restricted basal and the 30% molasses treatment. The total efficiency with which the DE was converted to milk energy and weight change followed the same trend, with the restricted basal and the 30% molasses treatments being significantly lower than either the basal or 10% molasses. The partial efficiency of energy utilization for milk production was quite different, however. When the cows received the restricted basal, milk energy yield showed a tendency to remain higher than expected, the energy yield being maintained at the expense of a weight loss. This resulted in a higher rate of conversion of DE to milk energy.

The partial efficiency of conversion of DE to milk energy is considerably lower than might be expected. Brody (3) cites the average efficiency of conversion of DE to milk energy of good cows to be approximately 33%. This value

TABLE 2

Composition of rations, as fed

Ration	Dry matter	Crude protein	Gross energy	Digestible crude protein	DE	TDN
	(%)	*(%)*	*(kcal/g)*	*(%)*	*(kcal/g)*	*(lb/100 lb.)*
Basal	89.7	18.0	4.14	14.1	3.15	70.1
Basal plus:						
10% Molasses	88.5	16.3	3.94	12.1	2.99	68.2
30% Molasses	86.1	13.1	3.73	8.9	2.83	65.3

TABLE 3

Energy yield and efficiency of energy utilization

Category of interest	Ration			
			Basal plus:	
	Restricted basal	Basal	10% molasses	30% molasses
Mean body weight *(lb.)*	1,181[a]	1,176[a]	1,182[a]	1,180[a]
Feed intake *(kg. per day, as fed)*	9.99	13.84	13.86	12.90
DE intake *(kcal. per day)*	33,766	46,779	46,015	40,764
Milk energy yield *(kcal. per day)*	8,498[a]	9,972[b]	10,537[b]	8,754[a]
Weight gain *(lb. per day)*	−0.44[a]	1.16[b]	1.10[b]	−0.15[a, b]
Energy equivalent to weight gain *(kcal.)*	−858	2,262	2,145	−261
Total energy yield (P) *(kcal. per day)*	7,640[a]	12,234[b]	12,682[b]	8,493[a]
Total energy efficiency *(%)*[c]	22.6[a]	26.2[b]	27.6[b]	20.8[a]
Partial energy efficiency *(%)*[d]	25.2[a]	21.3[b]	22.9[a]	21.5[b]

[a, b] Values in the same category with common superscripts are not significantly different by Duncan's multiple range test (4).

[c] $\frac{100 \times \text{total energy yield}}{\text{DE intake}}$.

[d] $\frac{100 \times \text{milk energy yield}}{\text{DE intake}}$.

was estimated by assuming 1,814 kcal. of DE per pound of TDN, estimating the TDN intake from average values and estimating the milk energy from the yield of 4% FCM. If one assumes that 1 lb. of TDN is equivalent to 2,000 kcal. of DE (14), the efficiency would be approximately 30 rather than 33%. Various factors could be responsible for the low efficiency of conversion of DE to milk energy of the cows in this study. Genetic ability, type of ration, and environment possibly all could be factors.

One of the conditions necessary for the calculation of NE according to the concepts presented in developing Equation (5) is that the mean body weight of the cows receiving each treatment must be essentially equal. This is necessary, that the maintenance requirements will cancel when comparing the various groups. This requirement was realized, as shown by the mean weights in Table 3.

The relative importance of the weight change in the estimation of total energy yield has a bearing on the accuracy of the determination of NE. The data reveal that the maximum contribution of the weight change was approximately 18.5% of the total energy yield on the full allowance of the basal ration. The mean energy equivalent of the weight change was approximately 8% of the total energy yield. An error in the estimation of this fraction of the total energy yield would, therefore, result in only a small error in the estimation of net energy.

Some observations on milk composition are presented in Table 4. The reduction in total solids caused by the addition of 30% molasses to the basal ration was due to a reduction both in butterfat and in solids-not-fat. The addition of 10% molasses, however, had no significant effect on total solids, fat, or solids-not-fat. The low butterfat on all treatments may be associated with the low roughage content of the ration, the sole source being pineapple bran. The lowered fat resulted in a significantly lowered heat of combustion of the

TABLE 4
Milk composition

Category of interest	Ration			
			Basal plus:	
	Restricted basal	Basal	10% molasses	30% molasses
Total solids *(%)*	11.38[a]	10.81[b]	10.76[b]	10.28[c]
Butterfat *(%)*	3.14[a]	2.50[b]	2.40[b,c]	2.15[c]
Solids-not-fat *(%)*	8.24[a,b]	8.31[a]	8.36[a]	8.13[b]
Milk energy:				
Kcal. per gram of solids	5.52[a]	5.31[b]	5.27[b]	5.21[c]
Kcal. per 100 g. fluid milk	63.0[a]	57.5[b]	56.8[b]	53.6[c]
Rancidity[d]	0.82[a]	0.91[a,b]	0.80[a]	1.15[b]
Flavor score[e]	35+[a]	35−[a]	35−[a]	33−[b]

[a, b, c] Values in the same category having common superscripts are not significantly different by Duncan's multiple range test (4).

[d] Mg. KOH to neutralize free acid in 1 g. of butterfat.

[e] Rancidity determinations and flavor tests were made by Dairymen's Association, Ltd., Honolulu. The mean flavor scores fell within the following ranges and descriptions:

Score	Description
36	Slight feed flavor
35–34	Distinct feed flavor
33	Unfit to bottle

milk solids from the cows on the 30% molasses ration. Because of the difference in the heat of combustion of butterfat and the solids-not-fat, it is to be expected that there would be a close relationship between butterfat and energy level of the milk. Such a relationship is presented in Figure 1. Each point represents the mean of four weekly determinations. The coefficient of determination (r^2) of 0.96 indicates that only 4% of the variation is unaccounted for. With the very high correlation and low error of the regression coefficient, it appears that this relationship is sufficiently accurate to be of predictive value over the range of approximately 1.5 to 4.0% butterfat.

Application of the prediction equation to the data of Perrin (12) indicates that it is reasonably accurate to about 6% fat. Above this level, the equation over-estimated the energy value. The mean predicted value was 6.14, whereas the observed value was 6.10 kcal. per gram of solids for 45 samples of milk averaging 5.05% butterfat. The prediction equation suggested by Perrin (12), based upon fat, nitrogen, and lactose content of the milk, also gave a value of 6.14 kcal. per gram of solids, but had the advantage of accurately predicting the energy value of samples containing more than 6% fat.

Although the rancidity levels shown in Table 4 are all somewhat high, there was a tendency for the milk produced on the 30% level to be somewhat higher than that produced on the other rations. The higher rancidity was undoubtedly partially responsible for the significantly lowered flavor score of the 30% molasses milk. The milk on all treatments scored somewhat low and is probably due in part to the low fat levels. It is noteworthy that the 10% molasses ration had no detectable effects either upon rancidity or upon flavor score. Mead and Dunkley (10) found that feeding 1 lb. of molasses 0.5 to 2 hr. before milking caused a slight feed flavor in the milk and that 2 to 3 lb. caused a distinct feed

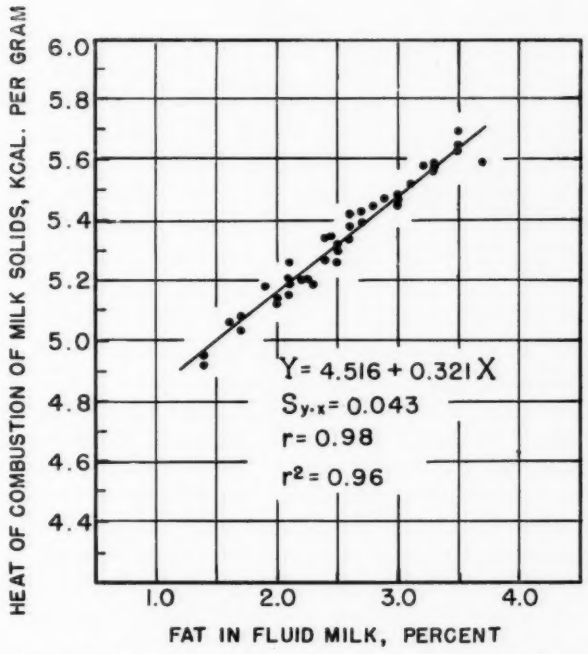

FIG. 1. The relationship of caloric value of milk solids to fat content of fluid milk.

flavor. The cows in these studies consumed an average of approximately 3.1 and 8.5 lb. of molasses per cow per day on the 10 and 30% rations, respectively. They were fed after milking, however, which would minimize the possibility of affecting milk flavor. Apparently, 3 lb. of molasses has no effect on flavor when fed over two feedings after the cows are milked. The larger amount of 8.5 lb., however, apparently affected milk flavor even when fed after milking. Other types of basal rations possibly could alter this effect.

The calculation of the NE of the basal ration for lactation is presented in Table 5 and for the two levels of molasses in Table 6. The value of 54.2 mcal. per 100 lb. for the basal is somewhat lower than would be calculated from Morrison's (11) estimated NE values for the individual ingredients. In the method used in this study, only the NE of the feed increment is measured. Any difference in the total NE of the restricted and the full allowance of basal thus is attributed to the increase in feed of the full over the restricted allowance of basal. Since the NE of a feed for maintenance is generally slightly higher than for production (6), one would expect the NE per 100 lb. of restricted basal to be higher than that of the full allowance. The NE of the feed increment would then have to be lower than either to account for the drop in NE. The NE of the

TABLE 5
Calculation of the net energy of the basal ration for milk production

Category of interest	Ration	
	Restricted basal	Basal
Total energy yield (P) *(kcal. per day)*	7,640	12,234
Feed intake (F) *(kg. per day)*[a]	9.99	13.84
Feed increment (F_1) [*kg.*(13.84 − 9.99)]	.....	3.85
Energy yield produced by F_1 [*kcal.* (12,234 − 7,640)]	.....	4,594
Net energy of basal ration:		
Kcal. per kg. (4,594/3.85)	.....	1,193
Meal. per 100 lb.	.....	54.2

[a] Feed intake is on an as-fed basis. See Table 2 for dry matter content.

increment thus can be considered closer to the true value of the feed for the process of lactation independent of maintenance.

For example, the lower level of NE required for maintenance of dairy cows given by Morrison (11) is described by the equation $NE = 0.0137\ W^{0.87}$, where NE is in meal. per day and W is in pounds. Using this equation, the maintenance requirement would be 6.45 and 6.43 meal. per day for the cows on the restricted and full allowance of basal, respectively. Adding to this, the milk energy yield and the energy equivalent of the weight change gives the total NE of the two treatments as 14.09 and 18.66 meal. per day. The daily feed intake was 22.0 and 30.5 lb. and the NE per 100 lb. of feed is, therefore, 64.0 and 61.2 meal. for the two rations, respectively. The feed increment of 8.5 lb. thus would have a NE of 54.0 meal. per 100 lb. to account for the drop from 64.0 to 61.2. In the method used herein to calculate NE, it is not necessary to know the maintenance requirement. The validity of the method is given support by the close agreement between the value of 54.2 meal. per 100 lb. of basal determined

TABLE 6
Calculation of the net energy of molasses for milk production

Category of interest	Ration		
		Basal plus:	
	Restricted basal	10% molasses	30% molasses
Total energy yield (P) *(kcal. per day)*	7,640	12,682	8,461
Feed intake (F) *(kg. per day)*[a]	9.99	13.86	12.90
Feed increment (F_1) *(kg.)*	.....	3.87	2.91
Molasses in F_1 *(% molasses × F)*	.....	1.39	3.87
Basal in F_1 *(F_1 less molasses in F_1)*	.....	2.48	−0.96
Energy yield produced by F_1 *(kcal.)*	.....	5,042	821
Energy in basal in F_1 *(kcal.)*[b]	.....	2,959	−1,145
Energy in molasses in F_1 *(kcal.)*[c]	.....	2,083	1,966
Net energy of molasses for milk production:			
Kcal. per kg.[d]	.....	1,499	508
Meal. per 100 lb.	.....	68.1	23.1

[a] Feed intake is on an as-fed basis. (See Table 2 for dry matter content.)
[b] Basal at 1,193 kcal. per kg. (See Table 5.)
[c] Energy yield produced by F_1 less energy in basal in F_1.
[d] $\frac{100 \times \text{energy in molasses in } F_1}{\text{molasses in } F_1}$.

in this study with the value of 54.0 mcal. per 100 lb. calculated by assuming a maintenance requirement.

The value of 68.1 mcal. per 100 lb. for molasses fed at 10% of the entire ration indicates that at this level molasses was more valuable than the basal ration as an energy source for lactation. The estimated NE value of molasses fed to dairy cows at not over 10% of the concentrate is 71.3 mcal. (11). The results of this study, therefore, show that blackstrap molasses may be fed in amounts up to 10% of the entire ration without materially reducing the NE from the level at 10% of the concentrate only. Increasing the molasses to 30% in the entire ration resulted in a three-fold drop in the NE of the molasses for lactation. One must conclude from this very large energy loss, together with the undesirable effect on milk composition, that with the type of ration used in this study, 30% molasses in the entire ration is too high for lactating dairy cows. The data obtained in this study are in good agreement with the values obtained by the authors with beef cattle (8). Molasses fed at levels of 10, 25, and 40% of the ration had NE values of 68.9, 37.8, and 35.1 mcal. per 100 lb. of molasses. It is somewhat surprising that the NE of molasses for fattening was as high as the values determined in this study for lactation. The basal rations were different, however, and it is known that NE, by reason of its critical nature, is subject to variation, depending on conditions of feeding. It is also true that the over-all efficiency of the dairy cows used in this study was low and could at least partially account for the NE of molasses for lactation to be no higher than that for fattening.

The present study does not indicate at what point the large energy loss occurs when molasses is increased from 10 to 30% in the ration. If the digestion trials are valid measures of the digestion losses of the cows on the lactation study, the loss must occur after absorption. It can be calculated from the data in Table 2 that the DE of the molasses in the 10 and 30% molasses rations is 2.13 and 2.23 kcal. per gram, respectively. Since there is no decrease in the DE on the 30% level, it is obvious that fecal energy can not account for the large loss in NE. It may be possible that the cows on the lactation study did not digest the higher level of molasses at the same rate as the steers on the digestion trial. There is a further possibility that the addition of high levels of molasses changes the proportion of fatty acids formed in the rumen. Armstrong and coworkers (1, 2) have shown that the individual fatty acids produce different heat increments when used to promote synthesis. Changing the fatty acid production to a high proportion of acetic acid could cause an increase in heat increment and a corresponding reduction in NE. The actual identification of the point of loss must await further investigation.

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AVAILABILITY AND METABOLISM OF VARIOUS SUBSTRATES IN RUMINANTS. I. ABSORPTION AND METABOLISM OF ACETATE[1]

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SUMMARY

Two experiments were conducted in which K-acetate-1-C^{14} was either injected or infused intravenously into a mature dairy steer to obtain measurements of pool size, turnover rate, and turnover time of blood acetate. Estimates were made of the amount of acetate absorbed and metabolized to carbon dioxide following feeding.

It was shown that a single injection of K-acetate-1-C^{14} is not applicable to the measurement of acetate pool size or turnover rate because of its apparent rapid metabolism before complete mixing with the unlabeled acetate in the pool is effected. However, the turnover time of the acetate pool was calculated to be 83 min.

The amount of acetate entering the pool after the consumption of 4.3 lb. of grain was estimated to be 67.24 g. per hour. Of this amount, 50% was oxidized to CO_2 and 50% was metabolized via other routes. The acetate oxidized accounted for 50% of the expired carbon dioxide.

Almost a century ago, it was observed that feedstuffs undergo fermentation in the rumen of cattle with the consequent formation of volatile organic acids. Few attempts were made to assess the significance of these acids in the nutrition of the ruminant until the early 1940's. Since then, numerous techniques have been devised to obtain a measure of the magnitude of the fermentation processes and to evaluate the importance of these acids to the nutrition of the host.

The use of in vitro techniques has contributed much to our knowledge of the origin of the volatile fatty acids and rumen gases; however, the cultural conditions imposed by the use of these techniques have without doubt brought about changes in the microflora and, consequently, shifts in fermentation patterns. Therefore, in vivo studies are necessary to evaluate the quantitative significance of isolated reactions or processes which have been observed in vitro.

The changes in relative concentrations of the volatile fatty acids in the reticulo-rumen contents of the intact animal after feeding have been used to measure the magnitude of the fermentation processes. Obviously, any change in rumen volume, rate of production, or rate of absorption would be reflected in a change in concentration of the metabolite under study. Estimates of the amounts of short-chain fatty acids arising during the fermentation of feedstuffs in vivo, arrived at by measuring changes in concentration, must be interpreted as indices rather than absolute values.

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[2] The data presented are from a thesis submitted by the senior author to the Graduate College, University of Illinois, in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

The isotope dilution technique has been used successfully to determine pool size, turnover rate, and turnover time of various metabolites in vivo. It appears that this technique might serve as a valuable tool in quantitatively measuring acetate absorption from the gastrointestinal tract of ruminants. However, before a true measure of acetate absorption can be made, it is necessary to have a knowledge of the possible sources of acetate and the amounts contributed by each.

The primary objective of the first study reported herein was to ascertain the magnitude of endogenous acetate production and concurrently to obtain estimates of pool size, turnover rate, and turnover time, using a single injection of acetate-1-C^{14} of high specific activity (5 mc/mM).

The objective of the second study was to measure the amount of acetate absorbed from the gastrointesinal tract of a fed animal, using the isotope dilution technique.

MATERIALS AND METHODS

The studies reported herein were conducted on a Brown Swiss steer weighing approximately 160 kg. The animal was fasted 72 hr. before conducting Experiment I. The major portion of the rumen contents was removed via a rumen fistula 15 hr. before injecting 1.25 mc. of K-acetate-1-C^{14} (prepared in 25 ml. of physiological saline) into the external jugular vein. Blood samples were taken at 15-min. intervals for the first hour, at 30-min. intervals for the second hour, and at hourly intervals for the next 4 hr.

Ten days before conducting Experiment II, a complete tracheal transection was performed on the steer, according to the technique of Colvin *et al.* (2). This permitted the separation of expired gases from eructated gases arising from fermentation in the rumen. The technique employed for the measurement and sampling of expired gases is described in detail in the subsequent paper (3).

A polyethylene tube[3] was placed in each external jugular vein prior to the start of the second experiment, by the technique of Ralston *et al.* (8). One tube served to draw blood samples from the animal and the other to infuse the labeled material into the blood by means of a constant infusion pump.[4]

Following a 24-hr. fast, a priming dose of K-acetate-1-C^{14} (267.2 μc. in 50 ml. of saline) was injected into the jugular vein and the infusion of labeled acetate was started immediately and continued for 7 hr. The infusion rate was 46.2 ml/hr of a solution of K-acetate-1-C^{14} (same as injected) containing 5.34 μc/ml. Blood samples were taken for acetate and glucose analyses at 30-min. intervals for the first hour and at hourly intervals thereafter. Expired gases were measured, sampled, and analyzed for carbon dioxide concentration and radioactivity.

The acetate-1-C^{14} used in these studies was synthesized via carbonation of methyl magnesium iodide. The yield based on barium carbonate-C^{14} used in the synthesis was 86%. The labeled acetate was assayed for purity and radioactivity by preparation of the silver salt, combustion to $C^{14}O_2$, and counting in an ion chamber, using a vibrating reed electrometer.

[3] Intradermic Polyethylene Tubing (i.d., .062 in.; o.d., .082 in.).

[4] Harvard Apparatus Company, Dover, Massachusetts.

Blood acetate was measured by a slight modification of the procedure of Neish (5). The volatile fatty acids in blood were prepared for chromatographic separation as follows: One vol. of heparinized blood (20-60 ml.) was laked in 2 vol. of water and the proteins precipitated with 1 vol. each of 10% sodium tungstate and 0.66 *N* sulfuric acid. The filtrate and washings were rendered basic with a few drops of saturated KOH and evaporated to dryness in a 60° C. water bath under reduced pressure. The residue containing the salts of the fatty acids was dissolved in a minimum amount of water (3-5 ml.) and acidified to approximately pH 2 with 10 *N* sulfuric acid. The acidified mixture was transferred quantitatively to a small beaker containing 3 g. of Celite 535 and mixed thoroughly. The Celite containing the sample was slurried with hexane and placed on top of a previously prepared column (8 g. of Celite 535 + 3.5 ml. of .2 NH_2SO_4). The acids were eluted with butanol-hexane soluents. Five-milliliter fractions of the eluate were collected and the acids titrated to a bromthymol blue end point with 0.01 *N* alcoholic KOH in a stream of CO_2-free air. When radioactivity measurements were desired, each 5 ml. fraction of eluate was transferred to a 5-dram screw-neck vial and the tube rinsed with 5 ml. of scintillation fluid (6 g. of 2,5-diphenyloxazole (ppo) per liter of redistilled toluene). The radioactivity was determined in a Tri-Carb Automatic liquid scintillation spectrometer[5] at −8° C., using a high voltage tap (810 v.) with discriminator settings of 10–50 and 50–100 v. Under these conditions, the counting efficiency was between 36 and 40%. After the radioactivity measurements, the acetic acid in the samples was determined by titration in the solvent-scintillation fluid

[5] Model 314X, Packard Instrument Company, La Grange, Illinois.

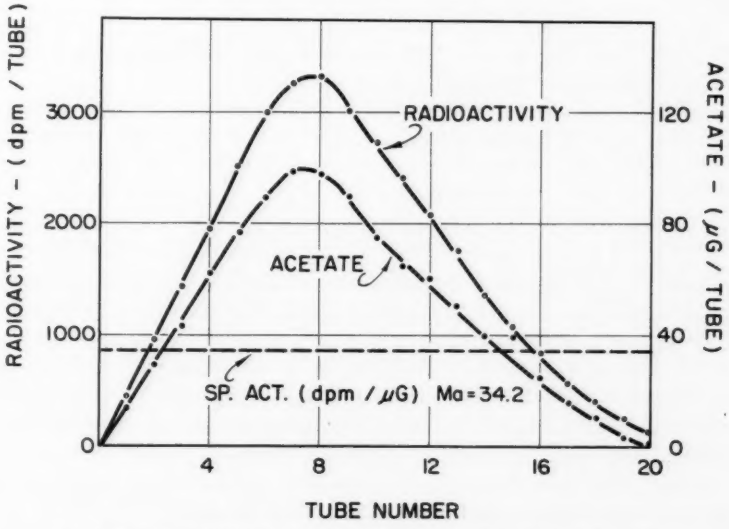

FIG. 1. Specific activity of blood acetate recovered from celite column.

mixture, as described. Calculation of the specific activity of the acetate in each tube gave a relatively constant value, indicating that the acetate fraction from the column was pure. These data are shown in Figure 1.

The carbon dioxide content of the expired air was determined, using a Model No. 35-802 Burrell apparatus. The remainder of the expired air aliquot was forced through an analytical absorption train in which the first two tubes contained 10 ml. of 0.5 *M* primene 81-R in absolute methanol (6) and the third tube contained 10 ml. of saturated KOH. To 5 ml. of the primene-$C^{14}O_2$ from the first tube was added 9 ml. of scintillation fluid (5 g. of ppo per liter of redistilled toluene). The samples were counted within 6 hr. in the same manner as was used for acetate-C^{14}, except that the best counting efficiency (40–48%) was obtained using a higher voltage tap (890 v.). Duplicate 1-ml. aliquots of the primene-CO_2 were titrated with 0.05 *N* NaOH to a thymolphthalein end point and the millimoles of CO_2 calculated after correcting for a blank titration.

RESULTS AND DISCUSSION

Experiment I. The terms, turnover time and turnover rate, are used as defined by Zilversmit *et al.* (12). Pool size denotes the amount of acetate which equilibrates with the injected isotopic acetate. The dilution of the injected dose measures the pool size.

To facilitate the discussion, a simple illustration is set forth in Figure 2. According to the diagram, the two possible sources of acetate are exogenous and

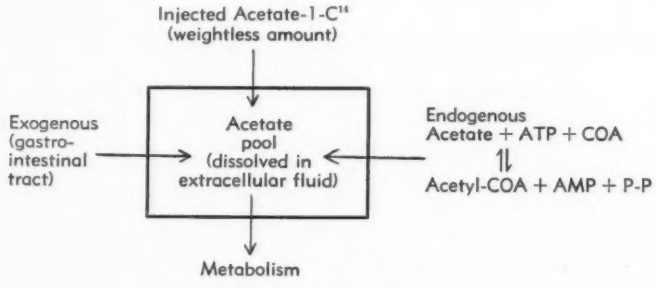

FIG. 2. Schematic diagram of acetate pool.

endogenous. It is well known that the former source contributes a significant amount of acetate under a normal feeding regime. The amount contributed from the latter source is unknown. The fact that prolonged starvation significantly reduces the acetate pool size, as evidenced by a sharp drop in blood levels of acetate (Figure 3), may be taken as an indication that the pool is maintained primarily by exogenous sources. This can not be interpreted to mean that the amount of acetate arising from endogenous sources is insignificant. If there is a high basic turnover of acetate, substantial quantities of this metabolite could be formed without showing a high concentration in any given tissue.

Through starvation and removal of the rumen contents, the amount of ex-

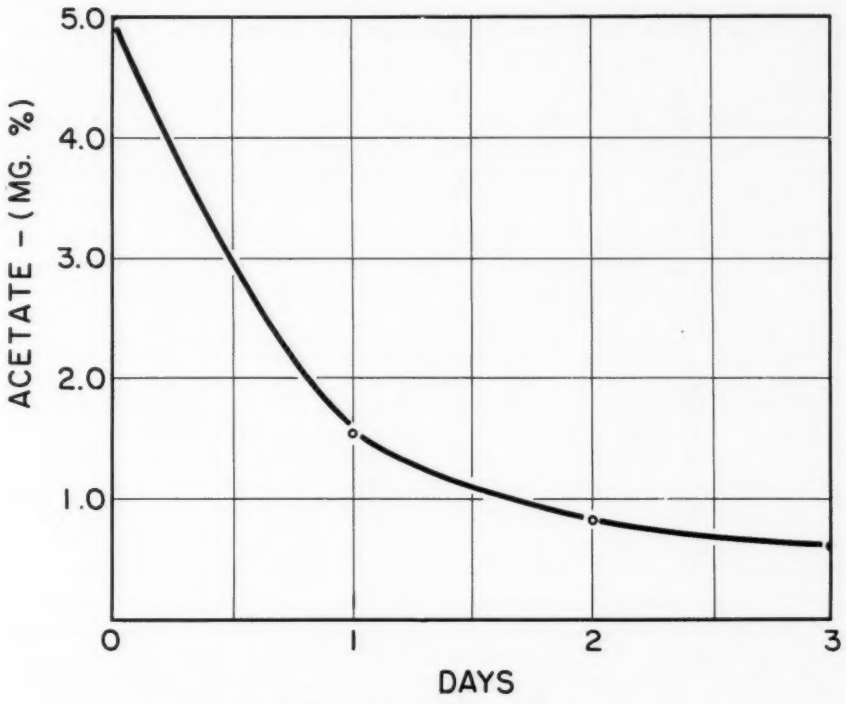

FIG. 3. Effect of starvation on blood acetate.

ogenous acetate was minimized, enabling a more accurate estimate of endogenous acetate production to be made. It is obvious from Figure 2 that if the exogenous acetate is eliminated, the only source of unlabeled acetate is from endogenous sources. Therefore, the decrease in specific activity of blood acetate after thorough mixing with the pool provides a measure of the amount of unlabeled acetate entering the system.

The results of Experiment I are presented in Figure 4. The drop in specific activity of the blood acetate during the first hour is attributed to the mixing of the injected labeled acetate with the unlabeled acetate present in the pool. From the slope of the curve, it appears that approximately 1 hr. is required for complete mixing to be effected. This finding is in agreement with that reported by Smyth (10), in which the experimental animals were cats. The decrease in specific activity between 90 and 150 min. is attributed to the dilution of the labeled acetate by the influx of unlabeled acetate into the pool. This portion of the curve appears to approach a first-order reaction indicating a steady state condition; however, more values for specific activity of the blood acetate would have to be obtained before this could be ascertained.

The isotopic acetate disappeared rapidly from the blood and was below the

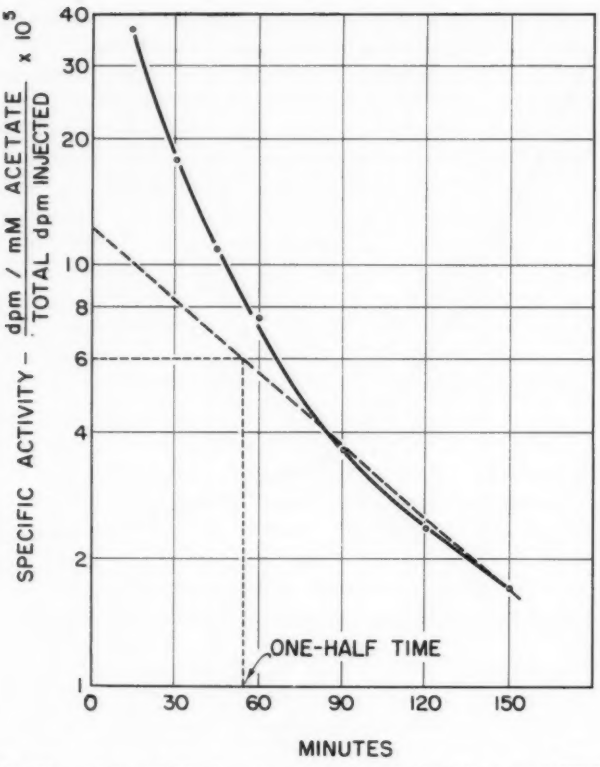

FIG. 4. Specific activity of blood acetate following single injection of $CH_3C^{14}OOH$.

level of accurate measurements after 150 min., even though 80 ml. of blood was used for analysis. These data substantiate the findings of Annison and Lindsey (1), that acetate-1-C^{14} administered intravenously to a fasted sheep disappeared rapidly from the blood. These authors calculated the half-life of the injected acetate-1-C^{14} in the blood to be 3.5–4.0 min.

The zero-time specific value was obtained by extrapolation of the log specific activity values from 90–150 min. back to zero time (Figure 4). Acetate pool size was calculated according to the following equation:

$$\text{Pool size (No. m}M\text{ acetate)} = \frac{\text{total disintegrations injected}}{\text{zero time specific activity of blood acetate}}$$

Specific activity at zero time is the theoretical value which would have been obtained if instantaneous mixing had occurred. Acetate space (the amount of fluid necessary to dissolve the pool so that the concentration is equal to that found in the blood) was calculated using the following equation:

$$\text{Acetate space (as per cent of body wt.)} = \frac{\text{m}M\text{ of acetate in the pool}}{\text{m}M\text{ acetate/100 ml. blood}} \times \frac{10}{\text{Body wt. in kg.}}$$

A specific gravity of one was assumed for all body fluids. When the above calculations are made using the data from Experiment I, an erroneously high value for acetate space is obtained. This value is of the order of 100 times larger than the animal. The probable source of an error of this magnitude is in the metabolism of the labeled acetate before complete mixing with the unlabeled pool of acetate was complete. As a result of the rapid utilization of labeled acetate before mixing had been completed, the specific activity of the acetate in the pool was lower than would have been the case if mixing had been instantaneous. If one assumes that acetate occupies the same extra-cellular space as does glucose (30% of body weight) (11), it is possible to calculate the theoretical specific activity of the pool acetate at zero time. These calculations are as follows:

$$\text{Specific activity } (t = 0) = \frac{\text{total disintegrations injected}}{\text{weight of animal (g.)} \times .30 \times \text{m}M \text{ acetate/ml}}$$

From this calculation, it was estimated that approximately 90% of the injected dose was metabolized before mixing was complete. This could account for all of the error incurred. The use of single injections of labeled glucose for the measurement of glucose pool size in dogs has yielded high values, which have been attributed to utilization of the isotope before mixing was complete (9).

The metabolism of the isotopic acetate before mixing was complete does not affect the measurement of half-time and turnover time of the acetate pool. Once mixing has been effected, the slope of the log specific activity versus time curve is changed only by the dilution effect of unlabeled acetate entering the pool, and not by metabolism. The half-time is read directly from the semilogarithmic plot (Figure 4). The turnover time is calculated using the following equation:

$$\text{Turnover time (Tt)} = T\tfrac{1}{2} \times \frac{1}{\ln 2}$$

Under the conditions of these experiments, the half-time was 58 min. and the turnover time 83 min. Popjack *et al.* (7) estimated the half-life of body acetate in goats to be approximately 50 min.

The significance of endogenous acetate production could not be determined, since a knowledge of turnover rate and pool size is necessary for the calculation of this value. However, the observed drop in the specific activity of blood acetate from 90 to 150 min. indicates that the amount of acetate entering the system was small compared to that entering from the gut under a normal feeding regime.

Experiment II. To reduce the error associated with the measurement of pool size and space, brought about by the metabolism of the injected isotope before mixing with the pool is complete, others (11) have resorted to a different manner of administration of the labeled material. A priming dose is given to shorten the time required to label the pool, followed by a constant infusion to maintain the specific activity of the substance. If the proper balance is achieved between the infused, labeled acetate and unlabeled acetate entering the system, the specific activity will remain constant after the initial mixing is complete. The decrease in blood acetate specific activity following feeding will give a measure of the

amount of acetate absorbed from the gut. Measurements of total expired gases, CO_2 content, and radioactivity of the CO_2 provide the data necessary to calculate the amount of acetate oxidized.

The specific activities of the expired carbon dioxide and blood acetate are shown in Figure 5. The specific activity of the expired CO_2 followed a trend

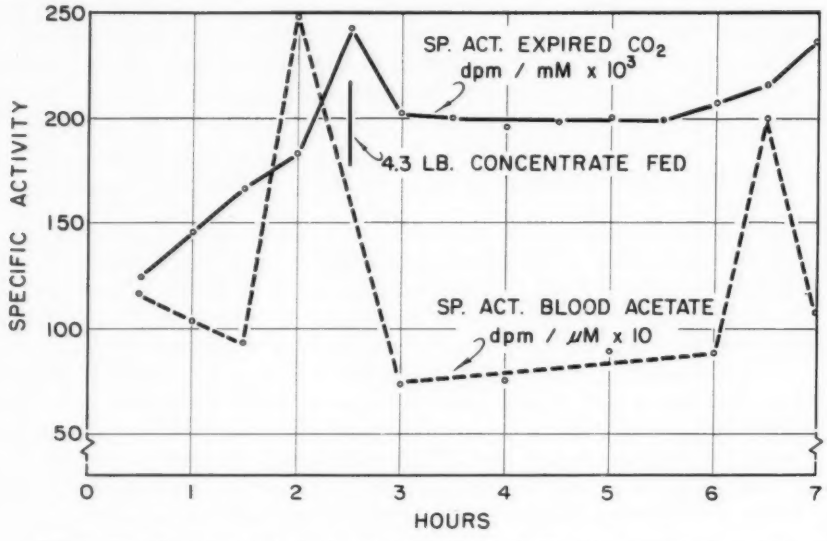

FIG. 5. Specific activity of blood acetate and expired CO_2 during constant infusion of $CH_3C^{14}OOH$.

similar to that of blood acetate. The high value for acetate at 2 hr. is difficult to interpret; however, the rise was real, as it was reflected in an increase in the specific activity of the expired CO_2. The difference in time between the two high values (acetate, 2 hr.—CO_2, 2.5 hr.) is probably due to the time required for the newly formed carbon dioxide to reach the lungs and be expired. The fact that the specific activities of the blood acetate and expired CO_2 were relatively constant from the third through the sixth hours indicates that the amount of acetate being oxidized to CO_2 was approximately the same for each 30-in. period. From the ratio of the specific activities (third through sixth hours), it is possible to calculate the per cent of the expired carbon dioxide derived from the oxidation of acetate. The equation is as follows:

$$\text{Per cent of expired } CO_2 \text{ derived from acetate} = \frac{\text{Sp. ac. of expired } CO_2 \times 2}{\text{Sp. ac. of blood acetate}} \times 100$$

The specific activities are expressed in the same units (d.p.m/mM); however, since the labeled acetate contained the isotope only in the carboxyl position, the specific activity of the CO_2 derived from the oxidation of this substance would

TABLE 1
Amount of acetate oxidized to CO_2

Time after start	Specific activity of Blood acetate	Specific activity of Expired CO_2	Expired CO_2 from blood acetate	Total CO_2 expired per hour	Theoretical amount of expired CO_2 from acetate	Acetate oxidized per hour
(hr.)	*(d.p.m/mM)*		*(%)*	*(mM)*		*(g.)*
3	726,000	200,432	55.2	2,134	1,178	35.34
4	750,000	197,800	52.8	2,267	1,197	35.91
5	894,000	199,621	44.7	2,286	1,022	30.66
6	882,000	218,455	49.5	2,186	1,082	32.46
Mean	813,000	208,077	50.5	2,218	1,120	33.60

be only one-half that of the acetate. Therefore, the observed specific activity of the CO_2 is multiplied by two.

From the total carbon dioxide expired, the theoretical grams of acetate oxidized is calculated as follows:

$$\text{Acetate oxidized (grams per hour)} = \frac{\text{m}M \text{ of } CO_2 \text{ from acetate per hour} \times 0.06}{2}$$

These data are shown in Table 1. The oxidation of acetate supplied the carbon for approximately 50% of the expired CO_2. This was equivalent to 33.62 g. of acetate. Since the blood level of acetate remained nearly constant at 3.0 mg. %, the amount of acetate entering the pool was equal to the amount being utilized (oxidation plus synthesis) by the tissue. During this time, the amount

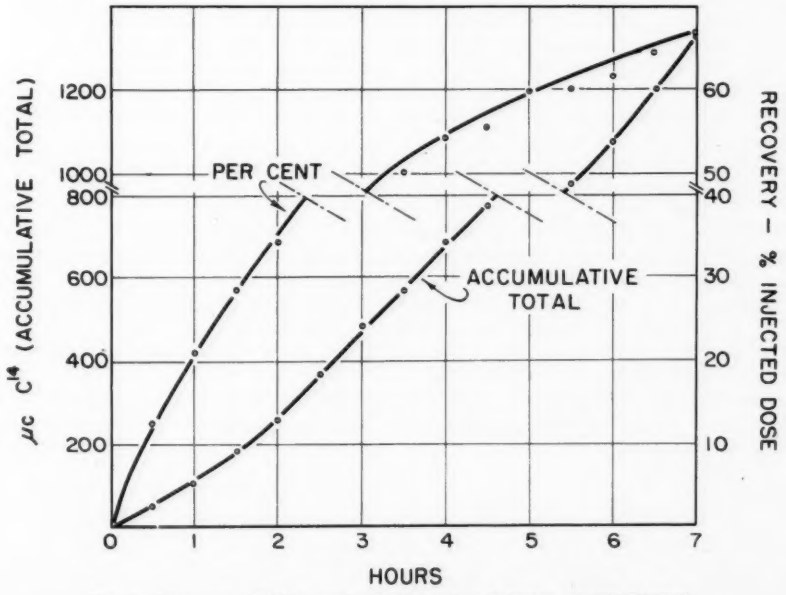

FIG. 6. Recovery of C^{14} in expired CO_2 during infusion of $CH_3C^{14}OOH$.

of the infused labeled acetate recovered in the expired CO_2 was approximately 50%. Therefore, the total amount of acetate entering the pool was twice the amount being oxidized, or 67.24 g/hr.

These data leave little doubt as to the significance of acetate in the general nutrition of the ruminant. If one assumes that the acetate oxidized was used efficiently and that the other substrate or substrates oxidized were at the same oxidation level as acetate, then acetate furnished approximately 50% of the total energy of the animal. An equivalent amount of acetate was available for other metabolic reactions.

From the results of Experiment I, it was evident that a large portion of the labeled acetate disappeared rapidly from the blood. The data of others (1, 4) suggested that a substantial amount of this could be accounted for in the expired carbon dioxide. Therefore, during this experiment, the per cent of infused dose oxidized to CO_2 was determined. These data are presented in Figure 6. Of the 1,994.8 μc. of C^{14} added as K-acetate-1-C^{14}, 1,333.8 μc., or 66.9%, was recovered in the expired CO_2 at the end of 7 hr.

ACKNOWLEDGMENT

The assistance of Dr. R. F. Nystrom in synthesizing the labeled material and in counting the samples is gratefully acknowledged.

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AVAILABILITY AND METABOLISM OF VARIOUS SUBSTRATES IN RUMINANTS. II. RATE OF ACETATE OXIDATION AS AFFECTED BY AVAILABILITY OF SUBSTRATE [1]

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SUMMARY

The sodium and potassium salts of acetic acid were infused into the external jugular vein of a 455-kg. Holstein steer at a rate to supply 20.27, 45.00, and 65.00 g. of free acetic acid per hour for a period of 7 hr. Acetate-1-C^{14} of high specific activity and of the same salt form was mixed thoroughly with the carrier (unlabeled) acetate before being infused.

The expired gases were measured, sampled, and analyzed for CO_2 content and their radioactivity determined. From the specific activity of the expired CO_2 and the total carbon dioxide output, the grams of infused acetate oxidized, as well as the rate of oxidation, was determined. Of the 142, 315, and 455 g. of acetate administered during the low, medium, and high rates of infusion, 103, 188, and 333 g., respectively, were oxidized to CO_2 and expired. The mean rates of acetate oxidation were calculated to be 15.46, 28.12, and 51.38 g/hr for the three rates of infusion, respectively. At the highest rate of oxidation (51.38 g/hr) acetate accounted for 34.2% of the total carbon in the expired CO_2. The contribution of the oxidized acetate toward the maintenance energy requirement of the animal was estimated to be 37.4%.

The technique used for the collection, measurement, and sampling of the expired gases is applicable to the study of factors affecting the oxidation of various metabolites to carbon dioxide. The simplicity of operation of the mechanical equipment and the normalcy of the animal under the experimental conditions make the technique extremely advantageous for studies of this type. The large size of the expired gas aliquot and the high counting efficiency of the $C^{14}O_2$ permit the use of low levels of radioactivity, thereby reducing hazard from radiation exposure.

The end products of microbial carbohydrate degradation in the rumen are, for the most part, short-chain volatile fatty acids (VFA). Absorption studies have demonstrated that large quantities of VFA become available to the ruminant (1, 5). More recently, the results obtained from rumen perfusion studies (4) indicate that the volatile acids are absorbed in the same relative proportions as they occur in the rumen. Acetic acid is present in the rumen in higher concentration than is any other volatile acid and also is more abundant in portal vein blood. The relatively low concentration of acetate in peripheral blood and the negligible excretion in the urine indicate that acetate is metabolized rapidly by the ruminant.

Few quantitative data are available concerning the rate of acetate metabolism in ruminants or the total amount of acetate the ruminant is capable of utilizing. The excellent work of Armstrong and Blaxter (2) depicts acetate as a metabolite

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[2] The data presented are from a thesis submitted by the senior author to the Graduate College, University of Illinois, in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

that is slowly and inefficiently utilized by fasted sheep. McClymont (6), on the other hand, found acetate to be utilized rapidly.

The studies reported here were undertaken to establish a maximum rate at which acetate could be metabolized and to measure the amount of acetate oxidized to carbon dioxide at various levels of acetate availability.

METHODS AND MATERIALS

The objectives of these studies required the development of a simple technique for measuring and sampling the expired gas from a mature steer. A schematic diagram of the experimental setup (Figure 1) shows the apparatus

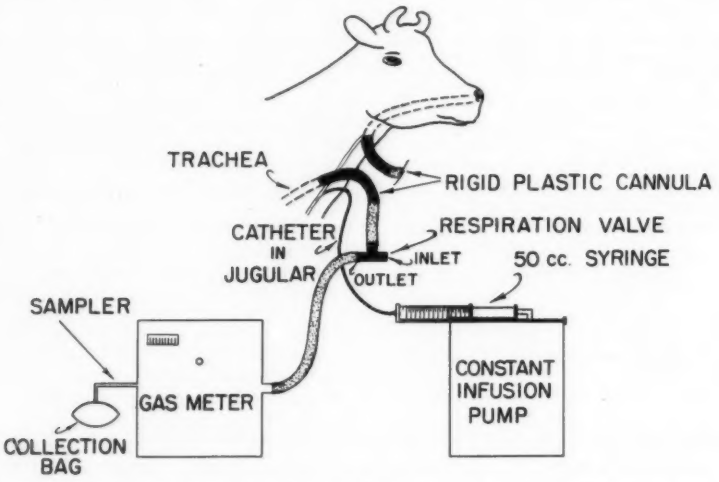

FIG. 1. Method for infusing metabolites and metering and sampling expired gas.

used to measure and sample expired gas. Previous studies (5) with two steers in which the tracheae had been transected revealed that in spite of careful postoperative treatment the cannulae were easily dislodged and tissue necrosis occurred in and near the ends of the transected trachea. For these reasons, complete tracheal transection was abandoned in favor of a method that is less drastic surgically.

The surgical field was infiltrated with 3% procaine hydrochloride and the trachea exposed by blunt dissection. A lateral slit was made in the trachea and a piece of cartilage was removed of sufficient size to permit insertion of a Dyson cannula (i.d., 1.0 in.). The self-retaining cannula was positioned in the trachea and the wound sutured, closed about the cannula. Appropriate antibiotic treatment was administered to prevent infection. Periodic removal of mucus from the cannula orifice is the only postoperative treatment required.

When expired gases were to be measured, an endotracheal tube (prepared from a 14 in. piece of gum rubber tubing, i.d., ½ in.—o.d., ¾ in.) fitted with an

inflatable cuff, was inserted caudally into the trachea via the cannula, and the cuff inflated. All of the respiratory gases passed through the catheter and contamination of the expired gas with eructated gas was eliminated. The exposed end of the endotracheal tube was connected to a three-way respiration valve[3] whose expiration port was joined to a portable respiration gas meter with an automatic continuous sampling device.[4]

A 455-kg. Holstein steer fitted with a Dyson tracheal cannula was used to obtain the data reported herein. After a 24-hr. fast, each external jugular vein was catheterized according to the method of Ralston *et al.* (10). One tube was used to obtain blood samples for glucose and acetate analyses, while the other was used to infuse the metabolite into the blood with the aid of a constant infusion pump.

Experiment I. K-acetate-1-C^{14} and nonlabeled reagent grade K-acetate were dissolved in sufficient distilled water to give a concentration of 0.8 g. K-acetate per milliliter, with a radioactivity of 0.828 μc/ml. This solution was infused into the blood at a rate of 41.4 ml/hr for a period of 7 hr. At this infusion rate, 33.1 g. of K-acetate (20.27 g. of acetic acid) was administered per hour. All results are calculated on the basis of free acetic acid.

Experiment II. The acetate solution used in this experiment contained 0.8 g. K-acetate per milliliter, with an activity of 0.582 μc/ml. This solution, infused continuously for 7 hr. at the rate of 92 ml/hr supplied 73.6 g. K-acetate (45 g. acetic acid) per hour.

Experiment III. The acetate-1-C^{14} used in this experiment was prepared as the sodium salt. An aqueous solution of Na-acetate-1-C^{14} and reagent grade Na-acetate was prepared which contained 0.4 g. of Na-acetate per milliliter, with a radioactivity of 0.427 μc. per milliliter. The amount infused per hour, calculated as the salt and as free acetic acid, was 88.8 and 65.0 g., respectively. The infusion was maintained for 7 hr.

During each experiment, the expired gases were sampled continuously and aliquots were taken at 30-min. intervals for 8 hr. The gas samples were assayed for carbon dioxide and radioactivity, as previously reported (5).

Nelson's method (9) was used to determine the glucose concentration in blood filtrate prepared by the method of Somogyi (12). Blood acetate was determined by a modification of the method of Neish (8), as described previously (5).

RESULTS AND DISCUSSION

The radioactivity of the expired CO_2 (Figure 2) increased during the infusion period, but dropped thereafter. The lower specific activity values observed during the early hours of the infusion period are attributed to one or a combination of the following factors: (1) the $C^{14}O_2$ derived from the oxidation of the isotopic acetate was diluted by $C^{12}O_2$ present in the bicarbonate pool,

[3] Two different valves were used. One was supplied with the gas meter (Footnote [4]); the other was obtained from Hans Rudolph, Kansas City, Missouri.

[4] Obtained from Max Planch Institute, Gottingen, West Germany.

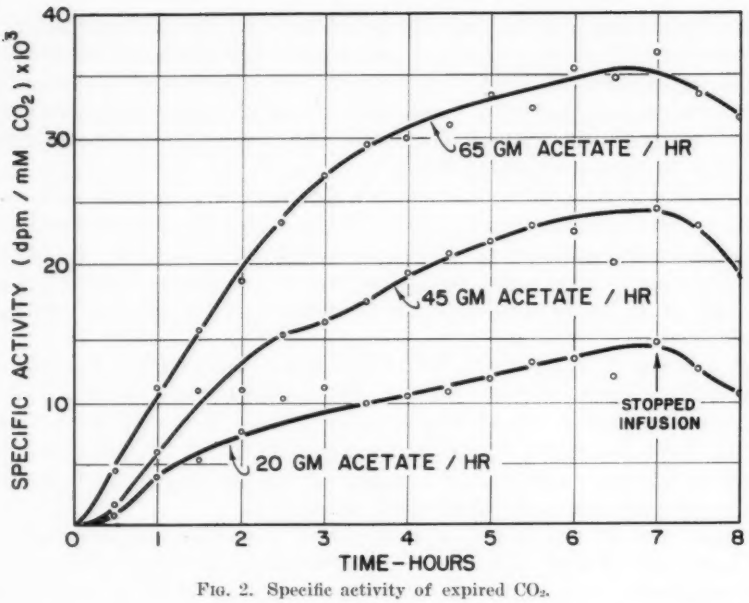

FIG. 2. Specific activity of expired CO_2.

(2) the isotopic acetate was diluted by the unlabeled acetate in the pool, thereby lowering the specific activity of the oxidizable substrate, or (3) the rate of acetate oxidation was lower at the start of the infusion and increased as time progressed. It is difficult to evaluate the significance of each of these factors; however, the first two are considered of major importance.

The amount of radioactivity infused per unit time varied in each experiment; therefore, the specific activity of the expired CO_2 was corrected for these differences. The corrected or relative specific activity values were calculated as follows:

$$\text{Relative specific activity of expired } Co_2 = \frac{\text{observed specific activity}}{\text{total disintegrations infused per minute}}$$

The relative specific activity curves (Figure 3) are practically identical, indicating that the fraction of isotopic acetate oxidized to $C^{14}O_2$ was the same, regardless of the amount of carrier acetate added. These data are presented in Table 1, expressed as the per cent of the infused dose recovered in the expired CO_2 for each 30-min. period. Although the amount of carrier acetate infused varied from zero to 65 g/hr, approximately the same fraction of the isotopic acetate was oxidized to carbon dioxide. These data are in agreement with those of Wick and Drury (13), in whose study eviscerated rabbits were the experimental animals.

The rate of oxidation of acetate by the ruminant appears to be dependent upon its availability to the tissue. Reid (11) reported that the magnitude of acetate uptake by the tissues in the head of the sheep, as measured by arteriovenous differences, was dependent upon the concentration of the acid in the arterial blood. The higher the blood level of acetate, the greater was the uptake

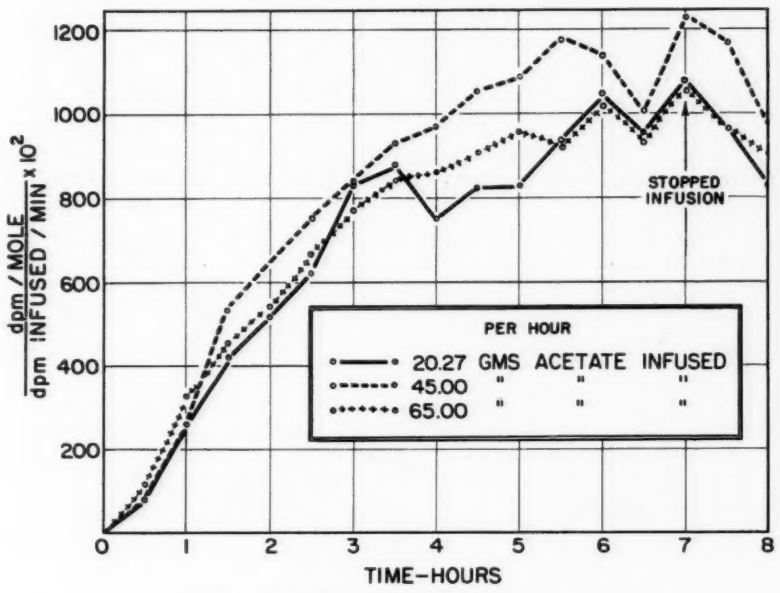

FIG. 3. Relative specific activity of expired CO_2.

of this acid. Glucose uptake was inversely related to acetate uptake. Under the conditions of the experiments reported herein, the rate of acetate oxidation was roughly proportional to the rate at which it was infused. These data are

TABLE 1

Recovery of infused dose in expired CO_2

Time	None[a]	Carrier acetate infused		
		20.27 g/hr	45 g/hr	65 g/hr
(hr.)		*(%)*		
0–0.5	12.6	6.4	5.4	8.7
.5–1.0	21.2	17.8	14.2	23.0
1.0–1.5	28.6	27.7	22.9	31.2
1.5–2.0	34.2	32.8	27.0	34.8
2.0–2.5	42.2	36.0	31.7	38.8
2.5–3.0	47.6	41.7	35.6	43.1
3.0–3.5	50.2	45.8	40.3	47.3
3.5–4.0	54.5	48.0	44.4	51.4
4.0–4.5	55.8	50.4	48.1	54.0
4.5–5.0	59.5	52.3	49.0	56.0
5.0–5.5	60.0	53.6	49.9	57.7
5.5–6.0	61.8	57.0	51.0	59.7
6.0–6.5	64.5	59.2	50.3	61.2
6.5–7.0	66.9	61.5	51.9	63.7
7.0–7.5	.....	67.2	55.7	67.4
7.5–8.0	.....	72.7	59.7	72.2

[a] Taken from other data in which a priming dose of acetate-C^{14} was given which was equal to the rate of infusion for 1 hr.

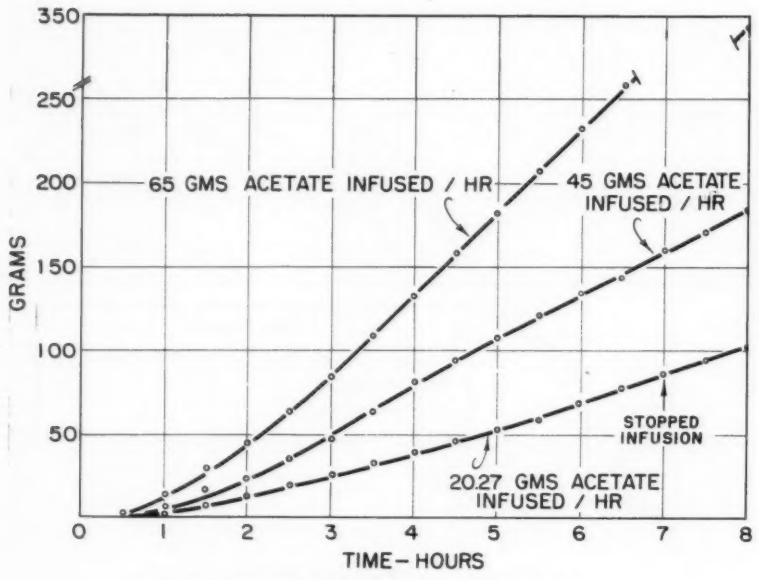

FIG. 4. Total grams injected acetate oxidized to CO_2.

shown in Figure 4. The total amount of acetate administered during the low, medium, and high rates of infusion was 142, 315, and 455 g., respectively. Of these amounts, 103, 188, and 333 g. were oxidized to CO_2 and expired at the end of 8 hr. Even at the highest rate of infusion (65 g/hr), the maximum capacity for acetate oxidation had not been reached. This is evidenced by the fact that the blood level of acetate was within physiological limits and the percentage of infused acetate oxidized to CO_2 was the same as that observed at lower rates of infusion.

The preceding figure shows the accumulative total grams of acetate oxidized to CO_2, but fails to show the changes in rate of oxidation as time progressed. The rates of oxidation of acetate (grams per hour) are presented graphically in Figure 5. It should be pointed out that the rate of acetate oxidation could have been constant throughout each experiment and yet show the trends as presented. Any factor which would cause the specific activity of the expired CO_2 to be low would tend to show a lower rate of acetate oxidation. These factors have been discussed previously. The values obtained between the third and the seventh hour are considered to be the true rates of acetate oxidation under these conditions. The mean rates of oxidation during this period for the low, medium, and high rates of infusion were 15.46, 28.31, and 51.38 g/hr, respectively. If one assumes a maintenance energy requirement of 11.56 therms per day of metabolizable energy (digestible energy $\times$ 0.82) (8), and that the infused acetate entered the energy scheme at the metabolizable energy level, then the

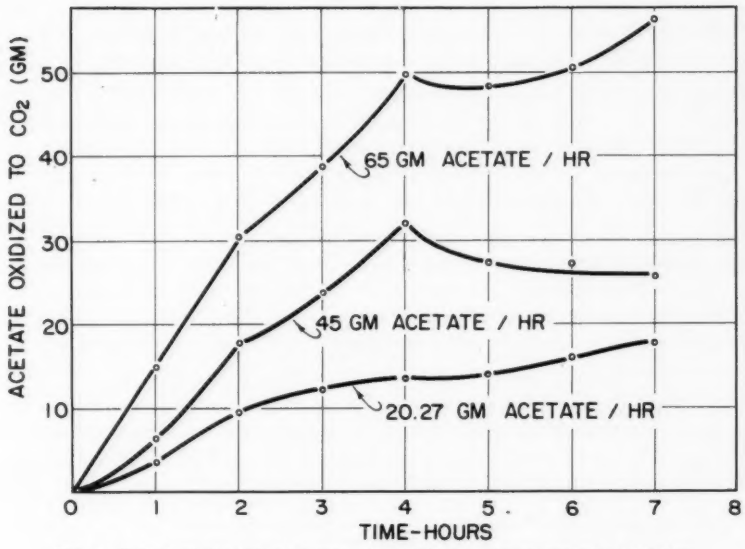

FIG. 5. Effect of the infusion rate of acetate on rate of acetate oxidation.

oxidation of 51.38 g. of acetic acid per hour (3.5 Cal/g) would supply 37.4% of the requirement, provided the system operated at this rate for 24 hr.

The fraction of expired carbon dioxide derived from acetate oxidation (Table 2) was calculated from the values obtained for the amount of acetate oxidized to CO_2 and the total quantity of CO_2 expired. These data show that as the oxidation of acetate increased, the amount of carbon dioxide derived from

TABLE 2

The per cent of the expired CO_2 accounted for by the oxidation of infused acetate

	Internal after start of infusion	Acetate oxidized to CO_2	CO_2 from acetate (theoretical)	CO_2 expired	Expired CO_2 infused derived from acetate
	(hr.)	*(g.)*	*(mM)*	*(mM)*	*(%)*
20.27 g. acetate infused/hr	3–4	13.54	451	5,060	8.9
	4–5	14.06	469	5,064	9.3
	5–6	16.28	543	5,098	10.7
	6–7	17.96	599	5,086	11.8
	Mean	15.46	516	5,077	10.2
45 g. acetate infused/hr	3–4	32.01	1,067	4,470	23.9
	4–5	30.24	1,008	3,763	26.6
	5–6	27.45	915	3,158	28.9
	6–7	23.52	784	2,990	26.2
	Mean	28.31	943	3,595	26.4
65 g. acetate infused/hr	3–4	49.53	1,651	5,383	30.7
	4–5	48.30	1,610	4,773	33.7
	5–6	50.76	1,692	4,797	35.3
	6–7	56.93	1,898	5,122	37.1
	Mean	51.38	1,713	5,019	34.2

other oxidizable substrates decreased. At the highest rate of acetate oxidation, 34.2% of the expired CO_2 was derived from this infused substrate. If one assumes that the other substrate (or substrates) being oxidized was at the same oxidation level as acetate, then the per cent of the total energy requirement furnished by acetate would be the same as the per cent of the total expired CO_2 derived from its oxidation. These values, 37.4 and 34.2%, respectively, are in close agreement.

The fact that the total CO_2 output was lower in Experiment II (45 g/hr) is attributed to the lower environmental temperatures and the quietness of the animal during the experimental period. The steer spent most of the period lying down and as a result the metabolic rate was reduced, as compared to the two other experiments, in which the environmental temperature was higher and the animal spent most of the time standing. The steer showed no signs of discomfort in the course of any of these trials, except when mucus collected in the endotracheal tube. The tube was then removed, cleaned, and reinserted. It was not necessary to clean the endotracheal tube more than twice during any experiment.

The blood levels of acetate and glucose are shown in Table 3. These values are considered to be within normal physiological levels. Armstrong and Blaxter (2)

TABLE 3

Effect of various rates of acetate infusion on the concentrations of glucose and acetate in the blood

	Acetate infused					
	20.27 g/hr		45.00 g/hr		65.00 g/hr	
Time	Glucose	Acetate	Glucose	Acetate	Glucose	Acetate
(hr.)	*(mg. %)*					
0	35.2	5.9	42.2	3.5	45.1	1.4
1	33.8	6.9	.....	.....	46.5	12.1
2	38.9	5.2	.....	.....	52.2	15.6
3	39.7	5.7	.....	.....	57.1	12.5
4	.....	....	.....	.....	53.6	9.6
5	36.8	4.5	.....	.....	57.8	11.0
6	36.8	5.6	....	.....	55.7	13.0
7	38.9	3.0	42.2	.....	53.6	10.4
8	42.6	1.7	40.0	14.8[a]	48.0	2.5

[a] Believed to be high, due to contamination from the syringe used to draw the blood sample.

reported significant changes in blood levels of glucose (decrease) and acetic acid (increase) following the administration of acetic acid into the empty rumen of sheep. The fact that there was no apparent drop in blood glucose or abnormal build-up of acetate in the blood during the studies reported herein suggests that the nutritional status of the animal plays a role in this phenomenon. The animals used by the English workers had been fasted for four days and the only exogenous source of energy was acetic acid.

Total urine output was not measured; however, it appeared that urination was more frequent when the highest level of K-acetate (45 g/hr) was infused. Analysis of urine samples collected at various times during the course of each experiment showed no appreciable amounts of acetic acid.

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INFLUENCE OF YOLK-CITRATE, YOLK-GLYCINE, WHOLE MILK, AND SKIMMILK EXTENDERS CONTAINING GLYCEROL ON MOTILITY OF BOVINE SPERM AT 37 AND 5° C.[1, 2]

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SUMMARY

Sixteen semen samples were extended 1:50 in 14 extenders with 7.5% glycerol added by direct and step-wise procedures to give 28 different extender treatments. Aliquots of each treatment were stored at 37° C. for 12 hr. and at 5° C. for seven days. Yolk-2.9% citrate, yolk-2.0% citrate, yolk-2.9% glycine, whole milk, and skimmilk were the basic extenders, together with mixtures of these at 1:1 in all possible pair combinations. Direct glycerolation generally gave better results than stepwise, as evaluated by progressive sperm motility, but these effects were not significant by seven days of storage at 5° C. Independent of glycerolation method, progressive motility at 37° C. was significantly higher in yolk-2.9% citrate and its combinations than in yolk-2.0% citrate and its combinations, but the reverse was true at 5° C. Extenders composed solely of milk constituents performed favorably at 5° C. and poorly with prolonged 37° C. storage. No single extender or combination of two basic extenders gave similar results, as time and temperature of storage and method of adding glycerol were varied.

Because the limit of economic use of semen extended in conventional egg yolk–citrate, whole milk, and skimmilk lies between one and three days, attempts have been made to prolong the life of sperm in these basic extenders. Most commercial and research departures from egg yolk–containing media aim at replacing the egg yolk with something less expensive or of simpler composition.

Certain additives to extenders have met with considerable success. Glycerol addition to extended fluid semen has lengthened sperm life (2, 3, 8) and increased the nonreturn rate (6, 7, 9, 16). Glycine in combination with yolk-citrate (2, 3, 4, 10, 11), whole milk, and skimmilk (2) increased the ability of sperm to maintain excellent progressive motility under storage at 5° C. In one study (17), addition of glycine to yolk-citrate gave unfavorable results.

Higher nonreturns at 60 to 90 days have been reported for extender mixtures of yolk-citrate-glycine-glucose (4) and glycerol-glycine-yolk-citrate-fructose (14). A mixture of skimmilk-yolk-glycerol resulted in higher motility ratings at 0-3° C. than yolk-citrate-glycerol or whole milk–glycerol (8). A combination of skimmilk-yolk-glycerol maintained sperm motility longer than skimmilk and glycerol or yolk-citrate and glycerol (12). Other reports show higher progressive motility for semen stored at 5° C. in mixtures of yolk-citrate, yolk-glycine, whole milk, or skimmilk with and without 7.5% glycerol, as compared with these extenders when not mixed (1, 2).

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[1] Scientific Paper No. 1792, Washington Agricultural Experiment Station, Pullman. Project 1107.

[2] The data are from a thesis presented by the senior author to the graduate faculty of the State College of Washington in partial fulfillment of the requirements for the Ph.D. degree.

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This study was conducted to compare several basic extenders singly and in combination, using two methods of adding glycerol and two storage temperatures (37 and 5° C.). Earlier studies indicate that physical and physiological properties are variable, depending on techniques and intended use of an extender (1, 2).

EXPERIMENTAL PROCEDURE

Sixteen semen samples, four from each of four dairy bulls on a weekly collection schedule, were used. Samples were placed in a 32° C. water bath until aliquots could be added (15 min. after collection) to 14 different extenders. Glycerolation treatments and storage temperatures used with each of the 14 extenders are described in Table 1.

TABLE 1

Glycerolation procedures and storage temperatures imposed as treatments upon each of 14 extenders

Basic extenders	Treatments imposed
14 basic extenders (see Table 2 for composition)	5° C. Storage with motility ratings at 0, 3, and 7 days. A. Direct glycerolation: Semen extended 1: 50 in 7.5 glycerolated extender, cooled to 5° C. in about 100 min. B. Stepwise glycerolation: Semen extended 1: 25 in nonglycerol portion; cooled to 5° C. in 100 min., 15% glycerol portion added at 5, 10, 15, and 20% of final volume at 100, 120, 140, and 160 min. after preliminary extension. 37° C. Storage with motility ratings at 0, 1, 3, 6, 9,[a] and 12[a] hr. A. Direct glycerolation: Semen extended 1: 50 in in 7.5% glycerolated extender, transferred to 37° C. bath. B. Stepwise glycerolation: Semen extended 1: 25 in nonglycerol; 15% glycerol portion added at 5, 10, 15, and 20% of final volume at 100, 120, 140, and 160 min. after preliminary extension.

[a] One rating person only; all other ratings by two persons (5).

The whole milk and skimmilk were prepared by the method of Thacker and Almquist (15), except that the milk was heated just to boiling and then cooled rapidly. Egg yolk was combined with either 2.0 or 2.9% sodium citrate or 2.9% glycine at a 1:1 ratio to make the yolk-citrate and yolk-glycine extenders. The whole milk and skimmilk, yolk-citrate, and yolk-glycine were mixed 1:1 in all possible pairs of combinations (Table 2), with all extenders containing 1 mg. streptomycin/ml extender.

The data were analyzed by analysis of variance, using individual degrees of freedom as outlined by Snedecor (13). The total of 895 degrees of freedom involved 28 extender treatments, two raters of motility, and 16 semen samples for each motility observation, time, and storage temperature. The interaction variance between extenders and method of glycerolation was used to test for significant differences involving individual extenders and method of glycerolation within extenders.

TABLE 2

Average percentage motility of semen in various types of extenders after storage at 37 and 5° C.

Time in storage	2.9% Sodium citrate				2.0% Sodium citrate										
	YC[a]	YC+WM[a]	YC+SM[a]	YC+YG[a]	YC	YC+WM	YC+SM	YC+YG	WM	WM+SM	WM+YG	SM	SM+YG	YG	Av.
37° C. storage						Group A—Direct addition of glycerol									
0 hr.[b]	46	51	46	47	47	47	47	48	50	48	49	39	50	41	47
1 hr.	43	46	44	43	45	45	45	45	46	42	46	38	45	37	44
3 hr.	38	42	40	39	40	40	40	38	38	38	42	29	41	37	38
6 hr.	30	39	34	36	31	28	28	33	24	23	36	20	38	20	31
9 hr.	28	30	25	28	19	14	14	22	9	9	24	6	26	19	20
12 hr.	17	10	9	16	8	6	6	15	3	1	11	0	8	9	8
						Group B—Stepwise addition of glycerol									
0 hr.[b, c]	46	52	52	49	46	51	51	53	53	50	49	48	49	50	50
1 hr.	45	47	45	45	40	48	45	47	49	50	47	44	46	45	46
3 hr.	42	43	42	34	33	40	40	34	35	38	41	33	38	19	36
6 hr.	32	36	35	30	27	33	31	27	23	23	27	17	29	12	27
9 hr.	25	22	20	20	17	19	13	16	10	10	19	6	19	2	17
12 hr.	17	6	9	6	7	8	7	8	1	2	7	0	10	0	6
5° C. storage						Group A—Direct addition of glycerol									
Day 3	27	26	24	32	26	30	30	36	40	34	35	31	37	14	30
Day 7	12	12	10	20	17	18	19	28	29	24	31	26	31	8	20
						Group B—Stepwise addition of glycerol									
Day 3	23	22	24	28	31	27	28	31	36	33	33	31	33	12	28
Day 7	10	10	13	21	18	18	16	24	30	24	32	25	34	12	20

[a] YC = yolk-citrate, WM = whole milk, SM = skimmilk, YG = yolk-glycine.

[b] Same values for 37 and 5° C.

[c] Extension 1 : 25 and no glycerol.

TABLE 3

Summary of tests for significant differences in progressive motility rating between extenders and methods of adding glycerol

Comparison	D.F.	Storage temperature						
			37° C				5° C.	
		0 Time	1 hr.	3 hr.	6 hr.	9 hr.	12 hr.	3 days	7 days
Glycerol (A vs. B)	1	L**	L*	H**	H**	H**	H*	H**	....
Extenders	13	**	**	**	**	**	**	**	**
Interaction	13	*	....	**	**	....	....	....	....
			Group A—Direct vs. Group B—indirect glycerolation						
YC[a]	1	....	....	....	....	....	....	H*	....
YC[a]WM	1	....	....	....	....	H*	....	H*	....
YC[a]SM	1	L**	....	....	....	....	....	....	....
YC[a]YG	1	....	....	H*	....	H*	H**	H*	....
WM	1	....	L*	....	....	....	....	H*	....
WMSM	1	....	L**	....	....	....	....	....	....
WMYG	1	....	....	....	H**	....	....	....	....
SM	1	L**	L*	....	....	....	....	....	....
SMYG	1	....	....	....	H**	H**	....	H*	....
YG	1	....	....	H*	H**	H**	....	....	....
YC[b]	1	L*	....	....	....	....	....	L*	....
YC[b]WM	1	L*	....	....	....	....	....	H*	....
YC[b]SM	1	L*	....	....	....	....	....	....	....
YC[b]YG	1	L**	....	....	H**	....	H*	H*	....
			Comparison of extenders						
All YC[a] vs. all YC[b]	1	....	....	H*	H**	H**	H*	L**	L**
YC[a]+YC[a]YG vs. YC[a]WM+YC[a]SM	1	L**	....	L*	L*	....	H*	H*	H*
YC[a] vs. YC[a]YG	1	....	....	....	....	....	H*	L*	L**
YC[a]WM vs. YC[a]SM	1	....	....	....	....	....	....	....	....
YC[b]+YC[b]YG vs. YC[b]WM+YC[b]SM	1	L*	L**	L**	....	....	....	....	H*
YC[b] vs. YC[b]YG	1	L**	L**	....	....	....	....	L*	L**
YC[b]WM vs. YC[b]SM	1	....	....	....	....	....	....	....	....
YC+Comb. vs. WM+SM+YG+Comb.	1	....	....	H**	H**	H**	H**	L**	L**
WM+WMSM+WMYG vs. SM+SMYG+YG	1	H**	H**	H**	....	....	....	H**	H**
WM+WMSM vs. WMYG	1	....	....	L*	L**	L**	L**	....	....
WM vs. WMSM	1	....	....	....	....	....	....	H**	....
SM+SMYG vs. YG	1	L*	....	H**	H**	H*	....	H**	H**
SM vs. SMYG	1	L**	L**	L**	L**	L**	L**	L*	L*

[a] 2.9% Na-citrate.

[b] 2.0% Na-citrate.

H = higher motility; L = lower motility and refers to the first-mentioned part of the comparisons.

* = Significant at the 5% level of probability.

** = Significant at the 1% level of probability.

RESULTS AND DISCUSSION

Table 2 shows the average progressive motility for semen in the different extenders at the various storage times and temperatures. Statistically significant differences are indicated in Table 3.

Storage at 37° C. At the start of incubation, sperm in the 28 extender treatments showed a range of 39-53% in progressive motility (Table 2). Milk-extended semen with both glycerolation treatments declined to essentially a nonmotile rating at 12 hr. Progressive motility of sperm in yolk-2.9% citrate was significantly higher than in yolk-2.0% citrate after 3 hr. Sperm in either of the yolk-citrate extenders alone and in combination with the milks and yolk-glycine showed higher average motilities at 9 and 12 hr., as compared with extenders not containing yolk-citrate.

Storage at 5° C. Motilities during storage at 5° C. showed differences between extenders unlike those observed for 37° C. storage (Tables 2 and 3).

After three but not seven days of storage at 5° C., motility of directly glycerolated semen was higher than that of stepwise glycerolated semen ($P < .01$). At 5° C. storage, the results resembled those previously shown by Albright *et al.* (2). Sperm motility in whole milk–yolk-glycine, skimmilk-yolk-glycine, and whole milk alone was generally higher than with other extenders ($P < 0.01$). Yolk-glycine resulted in less sperm motility than either of the yolk-citrate concentrations, but combining yolk-glycine and yolk-citrate gave very good results. In contrast with 37° C. observations, yolk-2.0% citrate was superior to yolk-2.9% citrate in maintaining sperm motility at three and seven days of storage at 5° C.

General discussion. This study showed striking differences between extenders in their maintenance of sperm motility at 37 as contrasted with 5° C. Reasonably, physiological limitations upon survival would not be the same at different temperatures. Utilization of available nutrients might, for example, be a limiting factor at 37° C., whereas temperature adjustments might be more critical at 5° C.

Emphasis needs to be placed on studies which characterize limitations to sperm survival at varying temperatures. Information is not available as to the influence of extenders upon sperm viability within the female reproductive tract. Carry-over effects of an extender are conceivable, even though there would be substantial dilution with uterine fluids. The successful field use of combinations of materials for semen preservation may be explained partially by their ability to provide optimum survival at different temperatures. However, no single extender or combination of two basic extenders in the present study gave similar results, as time in storage, temperature of storage, and method of adding glycerol were varied.

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INFLUENCE OF SEMINAL VESICULECTOMY ON CERTAIN PHYSICAL AND BIOCHEMICAL PROPERTIES OF BOVINE SEMEN[1,2]

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SUMMARY

A study was designed to investigate some of the changes in bovine semen characteristics following the extirpation of the seminal vesicles of four young bulls. Ejaculates from each bull were pooled on each collection day until a minimum of 5 ml. of semen was obtained. The pooling of ejaculates of each bull was necessitated by the number of tests conducted on each collection day. These characteristics were studied by physical evaluation techniques and biochemical analyses of pooled ejaculates collected before and after seminal vesiculectomy. The physical evaluations included the per cent of motile spermatozoa, the degree of progressive motility, semen pH, volume, concentration, and freezability. Biochemical analyses were conducted on semen fructose, semen citric acid, and five seminal amino acids—namely, glutamic acid, glycine, alanine, serine, and aspartic acid.

The removal of the seminal vesicles resulted in a 50% reduction in semen volume. Spermatozoan concentration and pH values were significantly higher in postoperative collections. The per cent of motile spermatozoa and motility ratings was significantly higher in preoperative collections. Seminal vesiculectomy did not affect the per cent recovery of live spermatozoa when frozen pre- and postoperative collections were compared.

Extirpation of the seminal vesicles resulted in extremely low levels of semen fructose and citric acid. Fructose was reduced to levels as low as 1.9 mg. % for a single collection, whereas citric acid values were reduced to levels as low as 0.7 mg. % for a single collection following glandular removal.

The mean concentration of the five individual seminal amino acids was not altered significantly by the removal of the seminal vesicles. Concentrations ranged from 1.51 to 6.06 mg. % prior to the operation, and from 2.36 to 6.72 mg. % after the operation. The concentrations in descending order were: glutamic acid, glycine, alanine, serine, and aspartic acid when preoperative collections were analyzed. Postoperative concentrations were in the same order, with the exception of a higher mean concentration of serine than alanine.

The secretory contributions of the accessory glands to the ejaculate of the bull have played an important role in semen physiological research. The essentiality of the secretion of the accessory glands is questionable. As early as 1900, Walker (17) demonstrated that mating and fecundity were possible after extirpation of the prostate or the seminal vesicles.

Differences found in the biochemical and physical properties of bovine semen can be partially attributed to the function of the accessory glands. Distinct ejaculate variations are noted in the volume of semen produced and the quantity of seminal constituents such as fructose, citric acid, and certain amino acids.

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The utilization of fructose by spermatozoa is perhaps the most important part of spermatozoan metabolism. Yamada (18), working with qualitative tests on human semen, was the first to identify fructose as the sugar of semen. Later, it was shown by Mann (9) that mammalian spermatozoa carry a small nutrient reserve and rely to a large extent on extracellular substances for metabolic processes. He was able to identify one of these substances as *D* (−) fructose.

Very little was known about the complex nature of semen until 1929, when Schersten (13) discovered citric acid in the seminal vesicular and prostatic fluids. Humphrey and Mann (6) postulated that even though citric acid and fructose are produced within the seminal vesicles, they are possibly produced by separate cells. Although the exact function of seminal citric acid is unknown, Mann and Parsons (10) have suggested that citric acid, combined with potassium and sodium ions, may aid in maintaining osmotic equilibrium in semen. It was also revealed by Lardy and Phillips (7) that citric acid partially influences spermatozoan motility.

The presence of various free amino acids in semen is due to the secretory function of the prostate, Marvin and Awapara (11). The seminal amino acids were found to be beneficial to spermatozoan motility through protection against ultraviolet and visible light, Giese and Wells (4), Tyler and Rothschild (15), and Lorenz and Tyler (8). Gassner and Hopwood (3), working with the chromatographic patterns of the seminal amino acids, found essentially five amino acids in the free form. These were identified as aspartic acid, glutamic acid, glycine, alanine, and serine.

This study was designed to determine some of the effects of seminal vesiculectomy of four young bulls on the physical and biochemical properties of bovine semen.

EXPERIMENTAL PROCEDURE

Physical evaluation. Four young, healthy bulls were selected for the purpose of seminal vesiculectomy. These included a 13-mo.-old Holstein, two 12-mo.-old Jerseys, and a 25-mo.-old Angus-Holstein crossbred bull.

The surgical extirpation of the seminal vesicles was accomplished through an incision made in the right sacral region of the bull. The glands were removed in the proximity of the seminal vesicular duct and as close to the ampulla as possible. The semen collection interval for the entire study was set at five days. At the termination of the study, two of the four bulls were slaughtered and their reproductive tracts removed. There were no visible traces of glandular structure surrounding the ampulla of the bulls.

The investigation included evaluations of six of the physical characteristics exhibited by bovine semen. The physical characteristics evaluated were microscopic evaluation of the per cent of motile spermatozoa, degree of progressive motility, ejaculate volume, spermatozoan concentration, semen pH, and semen freezability.

The microscopic evaluation of the per cent of motile spermatozoa was scored from 0–100, whereas spermatozoan progressive motility was rated from 0–10. The pH of the pooled ejaculates for each collection day was measured with a

Beckman pH meter within 45 min. after ejaculation. The spermatozoan concentration of each semen sample was determined by a Spencer bright-line hemacytometer, using the methods prescribed by Smith and Mayer (14). Ejaculate volume was recorded with a graduated test tube at the time of collection. Using freezability as an additional criterion for semen evaluation, each sample was diluted and frozen. In preparation for freezing, the freshly collected pooled ejaculates were diluted at the rate of 10:1 with a solution of 3.6% sodium citrate and 25% egg yolk before cooling to 5° C. Five to six hours after the initial dilution was made, the sample was rediluted with enough glycerolated extender to make a final concentration of 7.5% glycerol. After an 18- to 24-hr. equilibration period, the samples were placed in an alcohol bath at 5° C. The temperature was lowered, by the addition of dry ice, from 5° C. to −15° C. in 45 min. and as rapidly as possible thereafter. When the bath temperature exceeded −70° C., the samples were stored in a subzero mechanical refrigerator at −95° C. Each frozen semen sample was evaluated after one day of storage for the per cent of motile spermatozoa and the degree of progressive motility.

Biochemical analysis. Biochemical assays were conducted on the ejaculates collected before and after extirpation of the seminal vesicles. The assays included an analysis for fructose, citric acid, and five seminal amino acids conducted on pooled individual bulls' ejaculates collected every five days.

The procedure used for the analysis of fructose was similar to the methods described by Tyler (16). Semen collections were deproteinized and the fructose content analyzed within 45 min. after collection. Absorption was measured by a Klett-Summerson photoelectric colorimeter with a 420-mμ filter. Semen fructose concentration was calculated from the results of absorption readings from known fructose standards.

The procedure used for the determination of semen citric acid was suggested by Saffran (12). The citric acid analysis was initiated within 45 min. after collection. Absorption was measured by a Klett-Summerson photoelectric colorimeter with the use of a 540-mμ filter. Final semen citric acid was computed from the results of absorption readings from known citric acid standards.

Processing of semen collections for the quantitative determination of the five seminal amino acids was initiated 1 to 2 hr. after semen collection. The methods used for the analysis of the amino acids were modifications of chromatographic and elution procedures suggested by Block (1) and Giri (5). An ascending type of chromatographic separation was used. In preparation for analysis, the seminal plasma of a pooled series of individual bulls' ejaculates collected during one day was separated from the sperm fraction by centrifugation at 3,000 r.p.m. for 15 min. The volume of semen centrifuged was dependent on the volume of the series of ejaculates collected from the individual bull at that particular interval. An equal portion of 95% ethanol was added to the seminal plasma and thoroughly mixed. After hydrolysis, the supernatant was recentrifuged 15 min. and rehydrolyzed with another part of ethanol (v/v). The product was again centrifuged before the supernatant was removed and placed in a 5-ml. beaker. The supernatant was allowed to dry overnight in a warm-air oven main-

tained at 50° C. The dried residue was dissolved in a phosphate buffer (pH 6.2) and applied as spot applications on 8- by 3-in. strips of Whatman No. 1 paper. A series of three spots was made, three-fourths of an inch from the leading edge of the paper and at 1-in. intervals horizontally. Each spot was a composite of five sample applications of two Lambda volumes each. Between each of the five applications, the previous application was allowed to air-dry for 0.5-hr. All distilled water was boiled and the phenol solvent was redistilled to remove possible ammonia and heavy metal contaminants.

The 8- by 3-in. strips, containing the applied sample, were suspended approximately one-eighth of an inch into the redistilled phenol solvent, which was contained within an air-tight chamber. Following suspension in the solvent, the strips were removed and air-dried at room temperature for 24 hr. or until the odor of phenol was removed. The chromatograph was developed by spraying each strip with a 0.5% ninhydrin-acetone solution and allowing it to dry for 1 hr. The detected spots were eluted from the paper with a 4-ml. solution of 0.2 mg. of copper sulfate in 75% ethanol. Thirty minutes were allowed for extraction to take place.

The eluted spot was measured by an absorption reading obtained from a Klett-Summerson photoelectric colorimeter, using a 540-mμ filter. Final individual amino acid concentration was computed from the results of absorption readings from known amino acid standards which had been chromatographed previously.

RESULTS AND DISCUSSION

Postoperative ejaculates were collected one day after the removal of the seminal vesicles, to remove excessive clotting within the reproductive tract. Although temporary oligospermia existed in the first few postoperative collections, restoration of high concentrations of spermatozoa occurred within ten days after the operation. Postoperative collections were not used for evaluation or analysis until the semen appeared normal in concentration and per cent motile sperm.

In this experiment, a pooled series of ejaculates represented one collection. Since there were numerous semen evaluations and analyses to be made in the study, a minimum semen volume of 5 ml. was required of each bull on each collection day. Each bull was allowed to ejaculate without restraint, until this volume was obtained. The number of ejaculates required to obtain the volume of semen needed ranged from two to four before the operation, compared with two to seven after the glands were removed. Comparing the mean semen volumes obtained from each of the four bulls, before and after seminal vesiculectomy, there was a 49, 65, 42, and 40% reduction in semen volume. A comparison of mean values of all pooled ejaculates collected, before and after the operation, revealed a 50% reduction in semen volume per ejaculate obtained after glandular removal. Less reduction in semen volume was noted in the two youngest bulls. This may be attributed to the stage of seminal vesicle development in the younger bulls at the time of the operation. The interpretation of differences between the mean volume of semen and the mean concentration of spermatozoa of pre-

TABLE 1

Means of semen volume and ejaculates necessary to obtain 5 ml. of semen in pre- and postoperative collections[a]

Bulls	Age in mo.	Before operation* Av. ejaculates per 5 ml.	Before operation* $\bar{x}$ volume	Before operation* S_x volume	After operation Av. ejaculates per 5 ml.	After operation $\bar{x}$ volume	After operation S_x volume
1	25	2.45	3.06	.327	3.00	1.49	.392
2	13	2.50	3.30	.606	4.00	1.17	.208
3	12	2.90	2.34	.747	3.81	1.44	.562
4	12	2.90	1.90	.550	4.00	1.31	.625

* Collections made before operation were significantly higher in milliliters of semen per collection than after the operation ($P < .05$).

[a] These data represent a total of 11 collections from each bull before the operation and 13 collections obtained after glandular removal. A five-day collection interval was used.

and postoperative pooled ejaculates may be difficult, since more ejaculates had to be collected after the operation to obtain the minimum 5 ml. of semen necessary to conduct the series of tests in the study. The means of semen volume and the number of ejaculates required for 5 ml. of semen are shown in Table 1.

The means of spermatozoan concentration of collections before and after removal of the seminal vesicles are shown in Table 2. When the spermatozoan concentrations of each bull's pre- and postoperative collections were compared, an increase in concentration was noted in semen samples obtained after the operation. The difference in the spermatozoan mean concentration of each bull's collections, before and after the operation, was tested by the "t" test. The spermatozoan concentration was significantly increased in three of the four bulls by removal of the glands ($P < .01$). The major portion of this difference was due to the over-all decrease in postoperative seminal plasma. Although the semen volume was reduced to approximately one-half the volume obtained prior to the operation, the spermatozoan concentration increased only 1.3 times instead of the expected double concentration. These results suggest a possible impairment of spermatogenesis due to the operation.

TABLE 2

Spermatozoan concentration[a] and pH determinations of semen collected before and after the operation

Bulls	Before operation $\bar{x}$ Sperm conc.	Before operation S_x Sperm conc.	Before operation $\bar{x}$ pH	Before operation S_x pH	After operation $\bar{x}$ Sperm conc.	After operation S_x Sperm conc.	After operation $\bar{x}$ pH	After operation S_x pH
1	947	188	6.56	.173	1,365**	311	7.55*	.479
2	782	267	6.64	.141	1,042**	438	7.67*	.371
3	610	266	6.67	.172	698	320	7.92*	.303
4	620	306	6.66	.195	804**	329	7.85*	.316

* There was no significant change in the means of semen pH due to the operation when individual bull differences were compared ($P > .05$). The mean differences of pH values of all bulls' collections were significantly higher following the operation ($P < .01$).

** Ejaculates obtained from Bulls 1, 2, and 4 were significantly higher in spermatozoan concentration of postoperative collections ($P < .01$). Ejaculates obtained from Bull 3 were not significantly higher in spermatozoan concentration after the operation ($P > .05$).

[a] Represents a mean spermatozoan concentration from 11 preoperative collections and 13 postoperative collections. The mean concentration values listed are expressed in 10^6 sperm/ml.

The mean semen pH values for the four bulls represented increased significantly from 6.3, prior to the removal of the seminal vesicles, to 7.76 after the operation ($P < .01$). The postoperative ejaculates were characterized by extremely high pH values (as high as 8.43). Although these levels decreased to values as low as pH 7.2, the preoperative levels were not restored. Increases in semen pH values after the operation may be attributed to a percentage increase in alkaline buffer salts produced by the prostate and the bulbourethral glands, or possible disturbances in glandular secretory tissue due to surgical impairment. The pH values obtained from pre- and postoperative collections are shown in Table 2.

Microscopic evaluations of the per cent of motile spermatozoa and the degree of progressive motility, shown in Table 3, were made on diluted semen samples zero, one, three, five, and seven days of storage following collection of pre- and postoperative ejaculates. Freshly collected semen was diluted with a 25% egg yolk–3.6% sodium citrate solution at the rate of 1 : 10. Each diluted sample was stored at 5–6° C. until evaluation. The evaluation means of the percentage of motile spermatozoa before the operation were significantly higher ($P < .01$) than the evaluation means of the percentage of motile spermatozoa after the removal of the seminal vesicles. The means of spermatozoan motility ratings of ejaculates obtained before the removal of the seminal vesicles were not significantly higher for one-day evaluation ($P > .05$). Three, five, and seven-day evaluation means of spermatozoan motility ratings were significantly higher before than after the removal of the seminal vesicles ($P < .05$). Although the per cent of motile spermatozoa and the motility ratings were higher in preoperative collections, these data indicate that the dilution of freshly collected ejaculates in vitro may nullify some of the effects of seminal constituents, since large differences did not occur.

Since the freezing of semen is a common procedure in artificial breeding, freezability was used as one of the criteria for evaluating the quality of the ejaculates. Spermatozoan recovery rates, presented in Table 4, were computed for the per cent motile sperm and motility rates of the pre- and postoperative frozen semen samples. The differences between the evaluation means of the per cent of motile spermatozoa and motility ratings before and after seminal vesiculectomy were not significant ($P > .05$). Although a small number of samples were included in the study, there is an indication that seminal vesiculectomy did not affect recovery rates from freezing. This may be attributed to the fact that samples were in a frozen state within 24 hr. after collection.

Seminal vesiculectomy of the four bulls used in the study resulted in considerable change in the biochemical properties of the postoperative collections. The results were obtained from pooled individual bulls' ejaculates representing one collection day. The means of semen fructose of pre- and postoperative ejaculates are presented in Table 5. Extremes of 225 mg. % (lowest) to 291 mg. % (highest) were noted in the mean semen fructose concentration of the bulls' semen collected before removal of the seminal vesicles. After seminal vesiculectomy, the means of semen fructose for individual bulls' collections ranged from

TABLE 3

Comparison of the means of the per cent of motile spermatozoa and the degree of progressive spermatozoan motility evaluations[a] before and after the removal of the seminal vesicles

Semen age in days	Before operation								After operation							
	Bull								Bull							
	1		2		3		4		1		2		3		4	
	% Mot.*	Mot. rate	% Mot.*	Mot. rate	% Mot.*	Mot. rate	% Mot.*	Mot. rate	% Mot.*	Mot. rate	% Mot.*	Mot. rate	% Mot.*	Mot. rate	% Mot.*	Mot. rate
0	78	9.18	73	8.90	62	8.09	60	7.63	75	8.30	70	7.92	56	6.00	54	5.76
1	74	8.45	66	8.27	56	7.63	54	7.36	68	7.61	64	7.23	47	5.23	43	4.76
3**	63	7.91	58	7.63	45	6.18	45	6.27	57	6.69	56	6.30	37	3.30	28	2.92
5**	52	6.45	45	5.27	31	3.54	36	4.18	47	4.53	44	4.23	33	1.92	12	0.00
7**	35	3.90	20	2.63	10	1.45	20	2.36	27	3.07	18	1.76	8	0.00	2	0.00

* The mean difference of the per cent of motile spermatozoa was significantly higher on evaluations before than evaluations after the operation. ($P < .05$.)

** The mean difference betwen three-, five-, and seven-day evaluations (before) was significantly higher in spermatozoan motility than the three-, five-, and seven-day evaluations (after) at the .01 level of probability. The mean difference between one-day evaluations (before) was not significantly higher in spermatozoan motility than one-day evaluation after. ($P > .05$.)

[a] Comparisons were made from 11 collections prior to the operation with 13 collections after the operation. Microscopic evaluations were scored on a 0–100 basis for per cent motile and 0–10 for motility rating.

TABLE 4

Comparison of evaluations of spermatozoan motility of frozen semen collected before and after seminal vesiculectomy

	Before operation (44 samples)* $\bar{x}$	After operation (32 samples) $\bar{x}$
Initial per cent motile	68.1	63.9
Initial motility rate	8.5	8.6
Post-freeze per cent motile	45.7	41.7
Post-freeze motility rate	7.0	6.5
Per cent recovery motile sperm	62.5	63.6
Per cent recovery motility rate	79.0	74.0

* The differences between the means of post-freeze per cent motile sperm evaluation, before and after the operation, were not significant ($P > .05$). Differences between the means of post-freeze motility rates, before and after the operation, also were not significant ($P > .05$).

8.8 mg. % (lowest) to 12.2 mg. % (highest). Fructose levels, for individual samples, were found to be as low as 1.9 mg. % and as high as 27.4 mg. %. Although there was considerable variation of semen fructose in postoperative ejaculates, within the range of 1.9 to 27.4 mg. %, there was no indication of restoration to preoperative levels. Semen fructose found in postoperative ejaculates may be due to other glandular sources within the male reproductive tract. These data indicate that the seminal vesicles are the origin of almost all the fructose found in bull semen.

The results of the analysis of semen citric acid of pre- and postoperative ejaculates are shown in Table 5. The means of semen citric acid values for preoperative collections ranged from 210 mg. % (lowest) to 282 mg. % (highest) for individual bulls. Single-sample citric acid analyses ranged from a low of 0.7 mg. % to a high of 62 mg. % in postoperative collections. Although some vestigial semen citric acid existed, which may be attributed to other reproductive glandular sources, the significant decrease in semen citric acid after seminal vesiculectomy is indicative that almost all of the citric acid found in bull semen is produced within the seminal vesicles.

The quantitative analysis of five forementioned seminal amino acids was conducted 1 to 2 hr. following the collection of each series of ejaculates. It may be possible that delayed initial processing of semen influences the detection of

TABLE 5

Comparison of the mean semen fructose* and citric acid before and after the removal of the seminal vesicles (Expressed in milligrams per cent)

	Before				After			
	Fructose		Citric Acid		Fructose		Citric Acid	
Bulls	$\bar{x}$	S_x	$\bar{x}$	S_x	$\bar{x}$	S_x	$\bar{x}$	S_x
1	279	62.7	282	61.7	8.8	5.7	31.0	8.3
2	291	77.2	271	50.8	10.4	5.0	20.0	4.6
3	227	97.2	210	59.6	10.3	2.4	15.6	4.6
4	225	92.1	216	79.1	12.2	7.1	20.0	4.7

* Eleven fructose analyses and citric acid determinations were made from each bull before the operation and 13 analyses were made after the removal of the seminal vesicles.

differences in the amino acid content of bull semen, since Brown (2) has shown evidence that the amino acid concentration of human semen may increase to 100 mg. amino–*N*/100 ml. within 2 hr. after ejaculation. The results of the chromatographic analysis of five seminal amino acids found in seminal vesiculectomized bulls are presented in Table 6. From the collections obtained prior to

TABLE 6

Comparison of the means of five amino acid levels[a] from four individual bulls' ejaculates collected before and after the removal of the seminal vesicles

Amino acid	Mg. %—Before		Mg. %—After*	
	$\bar{x}$	S_x	$\bar{x}$	S_x
Aspartic	1.51	.65	2.36	.42
Glutamic	6.06	2.26	6.72	1.85
Serine	1.88	1.27	2.48	1.27
Glycine	4.94	3.96	5.18	3.78
Alanine	2.08	1.45	2.40	1.52

* No significant difference was noted between individual amino acid levels before and after the operation ($P > .05$).

[a] Comparisons were made from 11 collections before and 13 collections after the operation.

the removal of the seminal vesicles, certain seminal amino acid patterns were noted. The analyses revealed concentrations in descending order of glutamic acid, glycine, alanine, serine, and aspartic acid. The mean concentrations ranged from 1.51 mg. % (aspartic acid) to 6.06 mg. % (glutamic acid) before removal of the glands. Following seminal vesiculectomy, the amino acid pattern was the same with the exception of the position of serine. Seminal serine levels were of higher concentration in the postoperative collection than were alanine levels. Postoperative mean concentrations ranged from 2.36 mg. % (aspartic acid) to 6.72 mg. % (glutamic acid) after glandular removal. The mean differences of each individual amino acid between pre- and postoperative semen collections were tested by the "t" test. The test involved the difference between the means of the four bulls' pre- and postoperative collections for each amino acid. The mean difference between the individual amino acid level in the pre- and postoperative collections was not significant ($P > .05$). These results are more or less in agreement with the findings of Brown (2). The insignificant differences found between amino acid concentration of pre- and postoperative collections may be attributed to the actual delay in processing which could hamper the detection of more minute differences in concentration, or to the fact that the tissues involved in the production of the seminal amino acids were not disturbed by the surgical removal of the seminal vesicles.

An additional interesting observation was made in the course of studying the seminal vesiculectomized bull. It was noted that some of the secondary sexual characteristics of the three younger seminal vesiculectomized bulls were inhibited in development. This was a general opinion of researchers involved in the study as well as others unassociated with the project. The animals involved were noticeably sharp over the withers, had immature voices, and lacked the characteristic crests of bulls. The inhibition of these characteristics was apparent

in the younger bulls for 2 yr. after the operation. The 25-mo.-old Angus-Holstein bull possessed a well-developed crest at the time of the operation. No visible changes were noted in the older bull following the operation.

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LACTIC DEHYDROGENASE ACTIVITY IN BOVINE SEMEN[1]

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SUMMARY

Lactic dehydrogenase (LD) activity has been determined in 43 samples of bovine semen from 23 bulls of the Aberdeen-Angus, Holstein-Friesian, Guernsey, Jersey, and Milking Shorthorn breeds. The mean activity value expressed as Berger-Broida units/milliliter of bovine semen was 2,026 ± 151 units. Correlation coefficient between LD units/milliliter of semen and 30–60 day nonreturn conception data was −.414 ($P < 0.01$). Observations on 12 semen samples indicate that a considerable portion of LD activity is present in the seminal plasma.

The enzyme lactic dehydrogenase (LD) has been shown to be present in normal human blood plasma (16), in serous effusions, and cerebrospinal fluids. The activity of the enzyme in these fluids is especially high when they contain growing malignant cells. In like manner, the proliferation of neoplastic cells in tissue culture is associated with increasing LD activity in the culture medium (15). MacLeod and Wroblewski (9) observed that LD is present in high concentration in normal human semen and in seminal fluid of azoospermic individuals. They further observed that the LD activity of human seminal fluid was high compared to normal human blood plasma. A considerable portion of LD activity was present in sperm-free seminal fluid and appeared to be derived principally from the prostatic secretions.

Mann (10, 11) and Lutwick-Mann (12), utilizing ram semen, have presented evidence to indicate that the cozymase-catalyzed phase of fructolysis consists of two closely interwoven oxidoreduction processes. The first oxidoreduction involves the metabolism of phosphotriose through a series of enzymatic reactions and end products, with the ultimate production of pyruvic acid and adenosine triphosphate. The second oxidoreduction is between reduced diphosphopyridine nucleotide (DNPH) and pyruvic acid. DNPH is then oxidized to diphosphopyridine nucleotide (DPN) and pyruvic acid is reduced by lactic dehydrogenase (LD) to L(+)-lactic acid. The presence of fructose in bovine semen and the accumulation of lactic acid as a result of frutolysis are well known. The rapid cellular proliferation characteristic of bovine spermatogenesis, the presence of zinc in the prostate, seminal vesicles, and testicles of the bull (2), the presence of zinc in bovine semen (4, 5), and the presence of zinc in the molecule of LD suggested that LD activity might be present in bovine semen.

This investigation was conducted to determine the lactic dehydrogenase activity in bovine semen and to determine its relationship, if any, with certain semen characteristics.

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EXPERIMENTAL PROCEDURE

The lactic dehydrogenase (LD) activity of semen was estimated by the method of Berger and Broida (1), using 0.1 ml. of a 1:5 dilution of bovine semen. The theory of the method is based on the following reaction:

$$\text{Pyruvic Acid} + \text{DPNH} \xrightarrow{\text{LD}} \text{Lactic Acid} + \text{DPN}$$

Pyruvic acid reacts with 2,4-dinitrophenylhydrazine to form an intensely colored hydrazone which has a high optical density over the wave length range of 400-550 mμ. The wave length used in this investigation was 540 mμ. The amount of pyruvate remaining after the incubation of semen with pyruvic acid and DPNH is inversely proportional to the amount of LD present in the reaction. The LD activity is expressed as Berger-Broida units. The unit is equivalent to that amount of enzyme that would cause a decrease in optical density at 340 mμ of 0.001/min in a reaction mix of 3-ml. volume, as described by Wroblewski and LaDue (16).

Normal semen specimens were obtained from dairy bulls of the Arkansas Dairy Breeders organization. The semen samples were placed, unextended, in a walk-in type mechanical cooler maintained at 38° F., until all ejaculates to be processed were collected. A portion of each sample was then transported to the laboratory in a thermos bottle maintained at 36° F. The time between collection and assay of the sample for LD activity was about 1 hr. in each case. The concentration of spermatozoa was estimated by a photoelectric method similar to that of Willett and Buckner (14). Motility ratings were made by standard methods. Fertility data were obtained on the remainder of each sample from records of the Arkansas Dairy Breeders and were calculated on the basis of nonreturns at 30–60 days following insemination.

In 12 instances the LD activity was measured in whole semen, then semen from the same sample was centrifuged to separate the spermatozoa from the seminal fluid. The semen was transferred by means of a Wintrobe pipette into Wintrobe tubes and centrifuged at 1,600 r.p.m. on a 9-in. wheel for 60 min. Lactic dehydrogenase activity was then determined on the resultant cell-free seminal plasma.

RESULTS AND DISCUSSION

Lactic dehydrogenase activity values of 43 samples of whole semen from 23 bulls are presented in Table 1. The values ranged from 1,662 to 2,283 units/milliliter, with a mean activity of 2,026 ± 151 units. When these values are compared with those obtained in normal human blood plasma (range 200-500 units), it is quite obvious that the LD activity in bovine semen is very high. The values are lower than those found in human semen, where mean values of 5,600 units/milliliter have been reported (9). The mean LD activity in units/10^9 cells was 1,561 ± 614.

Fertility data were obtained on split ejaculate samples. The mean per cent of 30–60 day nonreturns from 1,110 first services was 77 ± 7. The correlation coefficient between LD units/milliliter of semen and 30–60 day nonreturn data

TABLE 1
Average concentrations of lactic dehydrogenase in semen

Breed	No. of samples	Semen characteristics: Volume of ejaculate	Semen characteristics: Initial motility	Semen characteristics: Concentration	Lactic dehydrogenase	
		(ml.)	*(%)*	*(thousands/mm³)*	*(units/ml)*	*(units/10⁹ cells)*
Aberdeen-Angus	3	5.00	54	1,270	2,013	2,068
Guernsey	10	4.95	80	1,513	2,099	1,592
Holstein-Friesian	16	5.94	72	1,534	2,025	1,375
Jersey	12	4.72	69	1,573	1,985	1,454
Milking Shorthorn	2	5.00	77	715	1,931	2,772
Mean		5.26	72	1,483	2,026	1,561
S.D.		1.42	17	500	151	614

was $-.414$ ($P < 0.01$). The correlaton coefficient between LD units/10^9 spermatozoa and 30–60 day nonreturn data was $-.071$ (not significant).

The correlation coefficient between LD units/milliliter and volume of semen per ejaculate was .131, and the correlation coefficient between LD units/milliliter and per cent motility at time of ejaculation was .050. These correlations were not significant.

In 12 samples, the spermatozoa were separated from the seminal plasma by centrifugation. The data pertaining to these samples are presented in Table 2. Mean LD activity of the seminal plasma (1,909 units), while lower than whole semen (2,120 units), suggests that a considerable amount of LD activity is present in the noncellular fluid component. Estimations based on cell volume determinations indicate that an average of $80.54 \pm 10.38\%$ of the LD activity was present in the seminal plasma. The mean cell volume percentage present was 10.2 ± 4.1. The mean concentration of spermatozoa in the 12 samples in 1,000/mm³ was $1,235 \pm 385$. The cell volume values agree very closely with values reported by some workers (3, 6, 8), are lower than one (13) and higher

TABLE 2
Concentration of lactic dehydrogenase found in whole semen and seminal plasma

Bull	Lactic dehydrogenase: Semen	Lactic dehydrogenase: Seminal plasma	Lactic dehydrogenase: Activity present in plasma	Semen characteristics: Cell volume	Semen characteristics: Concentration
	(units/ml)	*(units/ml)*	*(%)*	*(%)*	*(thousands/mm³)*
1	2,167	2,000	83.25	9.8	1,038
2	2,104	1,775	75.60	10.4	1,070
3	2,167	1,207	53.47	4.0	550
4	2,188	1,906	81.45	6.5	996
5	2,125	2,125	86.00	14.0	1,496
6	2,073	1,624	71.45	8.8	995
7	2,135	1,756	76.98	6.4	1,005
8	2,114	2,177	84.65	17.8	1,580
9	2,062	2,062	86.00	14.0	1,720
10	2,114	2,135	92.91	8.0	1,095
11	2,042	2,114	88.10	14.9	1,920
12	2,146	2,021	86.64	8.0	1,360
Mean	2,120	1,909	80.54	10.2	1,235
S. D.	44.7	281	10.38	4.1	385

than another (7). These differences may have been due to procedures involved. The coefficient of correlation between cell volume and the concentration of spermatozoa, as determined in this study, was .864. This value may be compared to values of .80 (13) and .917 (6) obtained by others.

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QUANTITATIVE DETERMINATION OF FREE ESTRONE, ESTRADIOL 17-β, AND ESTRADIOL 17-α IN BOVINE FETAL COTYLEDONS[1]

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SUMMARY

A quantitative method for assay of free estrone, estradiol 17-β, and estradiol 17-α in bovine fetal cotyledons is reported. Ethyl-acetate was used for initial extraction. The extract was partially purified by partitioning between toluene and 5% NaOH, followed by partitioning between the neutralized aqueous fraction and benzene. The estrogens were separated by paper chromatography, using the benzene-Skellysolve-B 1:1 formamide system. The estrogens were quantitatively determined by fluorimetry, using an adaptation of the method of Bates and Cohen. Recovery of exogenous estrogens added to fetal cotyledons was approximately 83% for each of the three estrogens studied.

Fetal cotyledons from 41 placentae of various stages of pregnancy were analyzed. Ten normal postpartum placentae averaged 3.5, 3.6, and 5.7 μg/100 g. of tissue, respectively, for estrone, estradiol 17-β, and estradiol 17-α, while total quantities present were, respectively, 55.9, 55.8, and 96.9 μg. Estradiol-17α was significantly higher than estrone or estradiol 17-β. In 31 prepartum placentae the concentration of estradiol 17-β was only slightly but consistently higher than either estrone or estradiol 17-α. The sum of the three estrogens present at 102-158, 170-212, 230-237, and 256-275 days of pregnancy was, respectively, 7.0, 11.2, 40.9, and 69.0 μg. while similarly the concentration per 100 g. of tissue was 1.4, 1.1, 2.4, and 4.0 μg. In contrast, postpartum fetal cotyledons averaged 12.8 μg/100 g. and the total present was 208.6 μg.

The placentae of several species are known sources of estrogenic compounds (9). Estriol, the principal estrogen in urine and placentae of humans, has not as yet been positively identified in the bovine (13, 21). Gorski and Erb (13) have established that estrone, estradiol 17-β, and estradiol 17-α are the principal naturally occurring free estrogens in bovine fetal cotyledons. The total quantity of these compounds increases during pregnancy (13, 22). Velle (21), using different extraction and separation techniques from Gorski and Erb (13), has shown that estradiol 17-α is present in greater quantities than estrone in postpartum bovine fetal cotyledons. Turner (19) has suggested that the rising level of estrogens during pregnancy eventually prevents uterine expansion and, thus, may be responsible for parturition.

Knowledge of estrogen levels in the bovine is relatively meager, primarily because assay methods adaptable to this species are not available. The primary purpose of the present study was to develop a routine chemical assay for free estrogens in bovine cotyledons. A secondary purpose was to use the method for preliminary study of levels of the free estrogens in bovine fetal cotyledons during various stages of pregnancy.

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EXPERIMENTAL PROCEDURES

Tissue sources. Fetal membranes were obtained from 41 cows ranging from 102 days of pregnancy to postpartum. Ten postpartum spontaneously released fetal membranes were obtained from normally calving cows in the Washington State University dairy herd. The gestations of these cows averaged 282 days and the standard deviation was five days.

Fetal membranes from 31 prepartum pregnancies were obtained at slaughter. Since conception dates were unknown, the stage of pregnancy was estimated using the crown-rump length of the feti (20).

Processing. All fetal cotyledons were immediately removed from the postpartum fetal membranes and either frozen at −25° F. or taken through the initial extraction. Either procedure appeared satisfactory. Facilities at slaughter did not permit removal of the fetal cotyledons prior to returning to the laboratory. Thus, for the 31 prepartum samples the uteri and their contents were placed in plastic bags and stored with solid carbon dioxide. Upon reaching the laboratory, partial thawing was necessary before the cotyledons could be separated from the fetal membranes and either initially extracted or stored at −25° F. These procedures for handling tissues are believed satisfactory, because Diczfalusy (7) has reported that estrogen content remains unchanged when placentae are in a frozen state. However, one can not ignore possible changes during the time required for freezing.

Chemical. The present procedure is modified from the procedure of Gorski and Erb (13) and consists of the following steps:

(a) Extract (100-g.) aliquots of cotyledons with 100 ml. of ethyl acetate three times in a Waring blendor. Pool the extract in a 1,000-ml. boiling flask by filtering through filter paper and Na_2SO_4 to remove H_2O. For postpartum and prepartum fetal cotyledons, 200 and 300 g. of tissue, respectively, are extracted in lots of 100 g., because a large-capacity blendor was not available.

(b) Ethyl acetate is removed from the pooled extracts under vacuum in a 45° C. water bath.

(c) The lipid-like residue is taken up in 10 ml. of toluene, transferred to a 40-ml. stoppered centrifuge tube, washed three times with 5-ml. aliquots of 5% NaOH by shaking approximately 100 times and centrifuging each time to hasten separation of the two solvents. The NaOH fractions are pooled and backwashed with 5 ml. of toluene to insure removal of all fats.

(d) The pH of the pooled NaOH fraction is lowered to 7 with 6 N H_2SO_4. A range of pH 7-9 is satisfactory for fetal cotyledons because interfering pigments are not a problem as is the case for urine (10).

(e) After cooling to room temperature, the neutralized aqueous fraction is extracted three times, using 15 ml. of benzene each time to remove the estrogens (7, 17).

(f) Benzene is removed from the pooled extract under water-pumped N_2 gas at 45° C.

(g) Estrone, estradiol 17-β, and estradiol 17-α are separated by paper partition chromatography (1, 13, 23) using benzene: Skellysolve B 1:1 (the nonpolar mobile phase) saturated with formamide. Whatman No. 1 chromatography paper (18½ by 22½ in.) is cut into 3-cm. strips, boiled in absolute ethanol for 15-30 min. to remove impurities, and air dried. The paper is then immediately saturated with a 50% formamide solution in methanol (23) and spotted at origin with the extract from fetal cotyledons. Descending chromatography is carried out in glass tanks for 11 hr. near 23° C.

(h) After chromatography, the chromatograms are air dried with a fan in the dark at room temperature for approximately 6 hr.

(i) Estrogens are detected by removing a 6-mm. strip and passing it through a v/v solution of 1% $K_3Fe(CN)_6$ and 1% $FeCl_3$, followed by washing in 5% HCl and distilled water (1). A permanent blue spot develops where estrogens are present. As little as 1–2 μg. of estrogens per sample can be detected by this procedure.

(j) The areas corresponding to the blue spots on the 6-mm. strips are cut from the 3-cm. strip. Elution is by dripping 8–10 ml. of redistilled methanol over the paper for 10–12 min. (12). In addition, an area showing no presence of estrogen is eluted to serve as a paper blank.

(k) After elution the eluates are concentrated to a volume of 5 ml. Eluates from prepartum fetal cotyledon extracts are divided into two equal parts of 2.5 ml. while 1-ml. aliquots are taken from the postpartum extracts. The remaining methanol in the aliquots is removed.

(l) The fluorimetric procedure of Bates and Cohen (2–4) was adapted for routine use. Braunsberg (5) reports this method to be more sensitive than the fluorimetric methods of Jailer (15) and Finkelstein (11).

Absolute ethanol in quantities of 0.2 ml. is added to the dried estrogen eluate because Bates and Cohen (3) found that precision of reading is increased when ethanol is present. A Coleman Universal Spectrophotometer Model 14 with a UV-1 and PC-2 filter combination is used. This instrument requires a volume of 10 ml. Therefore, instead of 1 ml. of 90% H_2SO_4 added to the alcoholic solution, as reported by Bates and Cohen (3), 1.4 ml. of 90% v/v H_2SO_4 is added. The mixture is heated for 10–15 min. in an 80° C. water bath. The tubes are removed after heating and 8.6 ml. of 65% v/v H_2SO_4 are added, giving a total of 10.2 ml. The solution is transferred to cuvettes and the fluorescence is measured. No background fluorescence was observed from either the ethanol, H_2SO_4, paper, or paper blank.

EXPERIMENTAL RESULTS

For convenience, the three estrogens will be referred to as E_1, $E_2\beta$, and $E_2\alpha$, respectively, for estrone, estradiol 17-β, and estradiol 17-α.

Recovery trials. Known amounts of the three crystalline estrogens were added to an aliquot of fetal cotyledons while another aliquot served as a control. Five trials early in the study gave average recoveries of 63, 56, and 63%, respectively, for E_1, $E_2\beta$, and $E_2\alpha$ (22). Similarly, the standard errors were 6, 5, and 4% (22).

A second series of recovery trials was made rather late in the present study, following refinements in the previously described methods. As shown in Table 1, a series of six trials had average recoveries of 83, 82, and 85%, respectively, for E_1, $E_2\beta$, and $E_2\alpha$. These compare favorably with recoveries reported by Diczfalusy (7) for free estrogens in human placentae. Further work has shown that 10 to 15% of the added estrogen is lost in the initial extraction of the tissue because when the estrogens are added to the pooled ethyl acetate extract recovery approximates 95%.

TABLE 1

Recovery of exogenous estrogens from bovine fetal cotyledons

Estrogen	Quantities present in control tissue	Amount added	Amounts recovered		S.E.[a]
	($\mu g.$)			(%)	
Estrone (E_1)	2.9	25	23.6	82.8	1.8
Estradiol 17-β ($E_2\beta$)	3.6	25	23.9	81.6	5.9
Estradiol 17-α ($E_2\alpha$)	6.7	25	27.3	84.6	1.1

[a] Standard error.

Chromatographic separation. Chromatography for a period of 11 hr. resulted in satisfactory resolution of the free estrogens isolated from fetal cotyledons. In ten trials the average distance moved by the compounds was 29.9, 11.7, and 8.0 cm., with standard errors of 0.5, 0.3, and 0.2 cm., respectively, for E_1, $E_2\beta$, and $E_2\alpha$. The 11-hr. period was the shortest time which resulted in suitable resolution of the compounds. Resolution of the three compounds is illustrated in Figure 1.

Fluorimetric analysis. The fluorescence exhibited by each estrogen from the extract was compared with the fluorescence of a known standard solution of each crystalline estrogen, to determine the amount of estrogen present. A 4-$\mu g.$ amount was used as the reference standard. Very early in the study it was noted that the fluorescence of crystalline E_1 was markedly decreased in the presence of paper eluate. Paper eluate had no effect on the fluorescence of $E_2\beta$ or $E_2\alpha$. Quenching due to traces of HCl or methanol has been reported by Diczfalusy (7). However, care was taken to remove all methanol by evaporation and HCl is not a factor in our procedure. The cause of quenching of estrone was traced to the chromatography paper, but the mechanism for the effect has not been found. With each set of fluorescence determinations, crystalline standards were added to a paper eluate blank and the fluorescence was developed and compared with the fluorescence of a standard solution. Throughout the study only E_1 was affected and in this case fluorescence was consistently decreased to one-half of that shown by a standard solution. The variation between samples was nearly always less than 6%. Since all comparisons of fluorescence were against the fluorescence of the standard solution, all values for E_1 were doubled to correct for the quenching effect of the paper.

Free E_1, $E_2\beta$, and $E_2\alpha$ in fetal cotyledons. Cotyledons on fetal membranes prior to 102 days of pregnancy were of insufficient volume for individual assay.

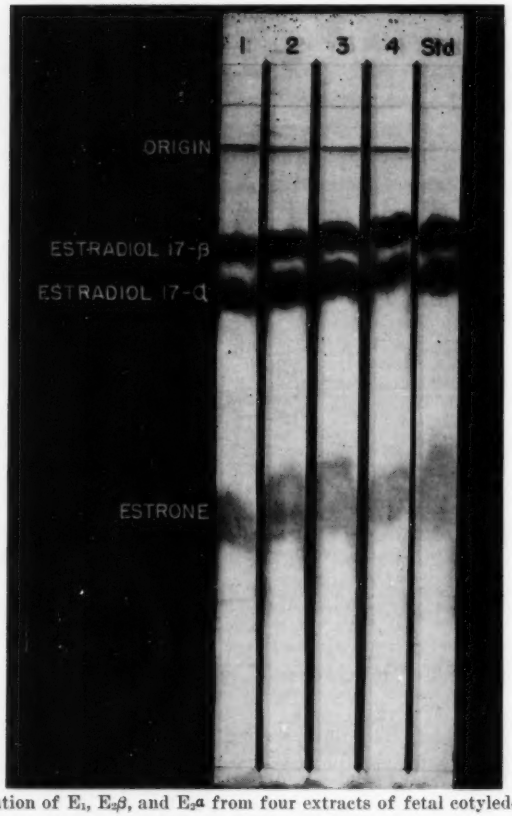

FIG. 1. Resolution of E_1, $E_2\beta$, and $E_2\alpha$ from four extracts of fetal cotyledons and the standard solution. Color was developed with $K_3Fe(CN)_6$ and $FeCl_3$ (1).

Thirty-one assays, made for prepartum stages of pregnancy ranging from 102 to 275 days, were grouped as shown in Table 2. Concentrations of E_1, $E_2\beta$, and $E_2\alpha$ were slightly higher for the group pregnant 102-158 days, as compared with 170-212 days. Concentrations of all three estrogens were higher by 230-237 days and E_1 and $E_2\beta$ showed a further increase for three samples taken at 256-275 days. The number of observations were too few to determine if differences among the prepartum groups were real. However, postpartum samples were significantly higher in concentration of all three estrogens, as compared with the prepartum samples as determined from the 95% confidence limits.

Table 2 also shows $E_2\beta$ was present in higher average concentration at each prepartum stage (not significant), while $E_2\alpha$ was significantly higher in postpartum samples than E_1 and $E_2\beta$ as determined by analysis of variance.

Though one can not predict any real changes in concentration of E_1, $E_2\beta$, and $E_2\alpha$ among the prepartum groups, there is nearly a tenfold increase in total quantities present, which was largely due to the increase in weight of the fetal

TABLE 2

Estrogens present in bovine fetal cotyledons at various stages of pregnancy

Stage of pregnancy	Samples	Estrone				Estradiol 17-β				Estradiol 17-α				Total estrogens			
		Total		per 100 g.		Total		per 100 g.		Total		per 100 g.		Total		per 100 g.	
			S.E.[a]		S.E.[a]		S.E.[a]		S.E.[a]		S.E.[a]		S.E.[a]		S.E.[a]		S.E.[a]
(Days)	*(No.)*	*(μg.)*															
102–158	10	2.00	0.41	0.42	0.05	2.82	0.90	0.50	0.10	2.16	0.43	0.48	0.09	6.98	1.45	1.39	0.15
170–212	12	3.25	0.46	0.35	0.15	4.08	0.69	0.39	0.17	3.83	0.57	0.37	0.14	11.15	1.04	1.11	0.07
230–237	6	11.44	1.50	0.67	0.03	15.64	2.65	0.93	0.16	13.87	4.35	0.81	0.26	40.93	6.74	2.42	0.40
256–275	3	19.26	2.10	1.15	0.16	25.36	3.81	2.04	0.20	16.20	8.82	0.82	0.28	69.00	14.04	4.01	0.01
Term	10	55.87	6.50	3.50	0.53	55.84	8.38	3.59	0.60	96.94	20.70	5.66	1.00	208.65	31.88	12.75	1.82

[a] Standard error.

cotyledons during pregnancy. The most striking increases in concentration and total quantities present were observed in the postpartum samples, as compared with the prepartum groups.

Considerable variation among individual postpartum samples was observed (see standard errors in Table 2). Some of this variation is likely due to breed of cow, since concentration and total estrogens in six Holstein samples averaged nearly twice as high as one Guernsey and three Jersey samples. Velle (21) also noted wide variation between postpartum samples of bovine fetal cotyledons and Diczfalusy (7, 8) made similar observations for human placentae.

DISCUSSION

Several workers (7, 9, 16) have studied the naturally occurring estrogens in human placentae, but until recently no workers have reported quantitative investigations of estrogens in bovine fetal cotyledons (13, 21, 22). Methods which have been developed for extraction of estrogens in human placentae are not entirely satisfactory for fetal cotyledons from cow placentae, because $E_{2\alpha}$ is present instead of estriol (12, 21). The method of Brown (6), which does not adequately separate the estradiols, was developed for and has attained wide usage for assay of human tissues and fluids which do not normally contain $E_{2\alpha}$. Doisy (9) reported the species differences between cows and humans as early as 1942. Gorski (12) was unable to detect the presence of estriol in bovine fetal cotyledons in limited studies carried on in this laboratory. Velle (21) was also unable to detect the presence of estriol in cow placenta. Because of these observations the present method was developed only for the extraction of E_1, $E_2\beta$, and $E_{2\alpha}$. It will not isolate estriol even if it is present.

The fetal cotyledons of the placenta were the only tissue analyzed in this study. Velle (21), in analyzing the inter-cotyledonary tissue areas, found only traces of free estrogens. He feels that the cotyledons are the only part worthy of analysis. Diczfalusy and Lindkurst (8) found an average of 4.7 μg. of E_1, 0.3 μg. of $E_2\beta$, and 12.5 μg. of estriol per 100 g. of human term placentae. Mitchell and Davies (17) reported much higher concentrations for human placentae than did Diczfalusy and Lindkurst (8). The difference appears primarily due to adjustment, according to recovery percentages by Mitchell and Davies (17). The average values for term bovine fetal cotyledons (Table 2) can best be compared with results from human placentae reported by Diczfalusy and Lindkurst (8), since recoveries of crystalline hormones were similar for the two methods (7) (Table 1), and no adjustments to reported recoveries were made. Ten samples of bovine cotyledons at term averaged 3.5 μg. of E_1, 5.6 μg. of $E_2\beta$, and 9.7 μg. of $E_{2\alpha}$ per 100 g. (Table 2). Comparatively speaking, more total free estrogens were found in human placentae. One can expect that the average per 100 g. of bovine placenta would be less than for fetal cotyledons, since Velle (21) found only traces of free estrogens in the inter-cotyledonary area.

Assaying the fetal cotyledons of bovine placentae appears to be an important step towards determining the role of estrogenic steroids in the reproductive processes in cows. Certain secondary evidence supports the theory that estrogens are produced in the placenta during pregnancy (7, 13, 18, 19).

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TECHNICAL NOTES

XANTHINE OXIDASE AND INCIDENCE OF SPONTANEOUS OXIDIZED FLAVOR IN MILK

In recent publications, spontaneous development of oxidized flavor has been attributed to catalytic activity of xanthine oxidase (1, 2) and of copper (9). Experiments described herein indicate that xanthine oxidase is not a limiting factor in determining whether oxidized flavor develops spontaneously.

Aurand, Woods, and Roberts (2) concluded that two different mechanisms exist for the development of oxidized flavor—that an enzyme, probably xanthine oxidase, is involved in development of spontaneous oxidized flavor; whereas, copper-induced oxidized flavor is a chemical oxidative process. Aurand and Woods (1) examined samples of milk from individual cows for xanthine oxidase activity and oxidized flavor development, and found a good correlation between these properties. Milks that possessed xanthine oxidase activity between 120 and 140 units (measured by a manometric method, results expressed as microliters of oxygen absorbed per milliliter of milk per hour) showed rapid flavor development; milks possessing an activity of less than 100 units failed to develop spontaneous oxidized flavor; and those with activity between 100 and 120 units were not consistent in the production of an oxidized flavor. Addition of xanthine oxidase to milks low in activity resulted in development of the flavor, as measured by the thiobarbituric acid method.

King and Dunkley (9) concluded that natural copper in milk is an important catalyst of spontaneous oxidized flavor when cows are maintained on an alfalfa hay–grain concentrate ration and are not in early lactation. They observed a highly significant correlation between natural copper and incidence of spontaneous oxidized flavor.

The following experiments were initiated after an earlier report by Woods, Aurand, and Roberts (13), but were completed before their more recent publications (1, 2). The procedures used in sampling milk have been described (9). The results of organoleptic examinations are expressed as flavor scores indicating intensity of oxidized flavor: 0, none; 1, questionable to very slight; 2, slight but consistently detectable; 3, distinct or strong; 4, very strong. Oxidized flavor was considered spontaneous if the samples, without added copper, received a score of one or more after refrigerated storage for five days. Xanthine oxidase was determined by two methods —a Thunberg tube technique (4, 12) and the vanillic acid method (8, 10). The Thunberg tube technique was based on anaerobic reduction of methylene blue at 18° C. with xanthine as substrate. Determinations were made in duplicate, at least. The procedure used for the vanillic acid method (8) differed from the published procedure (10) in several respects, but principally in the use of a lower incubation temperature (37° C.) to make the absorbance linear with concentration of xanthine oxidase. Xanthine oxidase activities by the Thunberg tube method are expressed as reciprocal of the time (seconds) required for 90% reduction of methylene blue, and by the vanillic acid method as microliters of oxygen absorbed per milliliter of milk per hour.

Data for xanthine oxidase activity determined by the Thunberg tube technique after refrigerated storage for at least 48 hr. are plotted against corresponding flavor scores for 41 milks from 32 individual cows (Figure 1). Similar data for 32 milk samples from 16

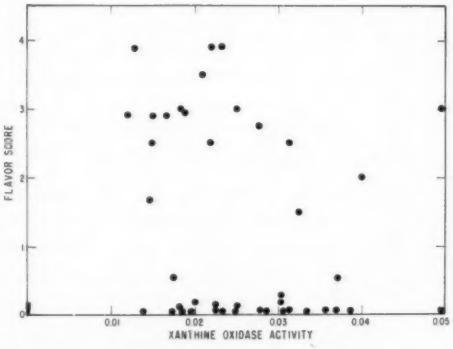

FIG. 1. Relation between xanthine oxidase activity measured by the Thunberg tube technique (1/seconds for 90% reduction of methylene blue, determined after refrigerated storage for at least 48 hr.) and spontaneous development of oxidized flavor during storage for five days at 3° C.

individual cows and two bulk milks based on xanthine oxidase determinations by the vanillic acid method were obtained over a different time interval (Figure 2). The distributions in these figures are not in agreement with the observation of Aurand and Woods of a good correlation between xanthine oxidase activity and spontaneous oxidized flavor.

The unit used to express xanthine oxidase activity by the vanillic acid method is the same as that used by Aurand and Woods (1). However, the results are not directly comparable because different substrates were used. Oxygen is not absorbed as rapidly with vanillin as substrate as with hypoxanthine or xanthine as substrate.

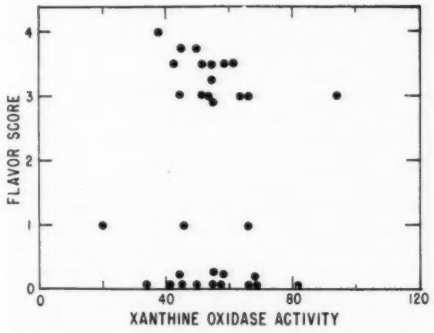

FIG. 2. Relation between xanthine oxidase activity measured by the vanillic acid method (in microliters of oxygen absorbed per milliliter of milk per hour, determined after refrigerated storage for at least 48 hr.) and spontaneous development of oxidized flavor during storage for five days at 3° C.

A possible factor that might explain the discrepancy with the observation of Aurand and Woods is the increase in xanthine oxidase activity during the aging of milk (5, 11). In results presented for two milks (Figure 3), typical of those determined for seven samples, most of the increase occurred in the first 12 hr. In 16 samples, including both spontaneous and susceptible milks, a direct relation was observed (Figure 4) between initial activity (determined within 2 hr. of milking) and final activity (determined at least 48 hr. after milking). Therefore, when the conditions of storage and the time of testing for xanthine oxidase are standardized

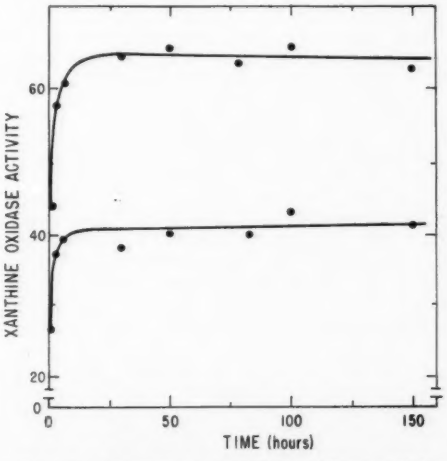

FIG. 3. Increase in xanthine oxidase activity of milk (vanillic acid method) during refrigerated storage (3° C.).

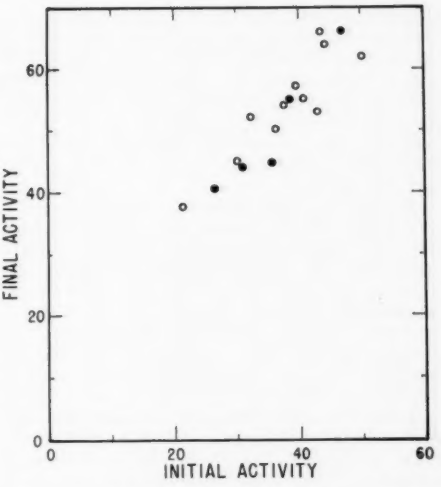

FIG. 4. Relation between initial and final xanthine oxidase activity (vanillic acid method). Initial activity was determined within 2 hr. of milking, and final activity after storage at 3° C. for at least 48 hr.
○—Spontaneous milk ●—Susceptible milk

(as for data in Figures 1 and 2), it is unlikely that the variation in xanthine oxidase activity during storage would influence a possible relation between xanthine oxidase and spontaneous oxidized flavor.

The experimental approach of Aurand and Woods (1), based on additions of xanthine oxidase to milk, also was used to investigate the relation of xanthine oxidase to spontaneous oxidized flavor. Two preparations of xanthine oxidase were used, one obtained from a commercial source[1] and the other prepared from raw milk by the method of Ball (3). As indicated below, interpretation of the results was complicated by the presence of three impurities in the xanthine oxidase preparations.

Milk samples to which xanthine oxidase was added developed a pronounced bitter flavor on storage and this made organoleptic evaluation of little value. Ball's method (3) for preparation of xanthine oxidase requires the use of a proteolytic enzyme (pancreatic lipase or crude trypsin), and ammonium sulfate to salt out and preserve the enzyme. Dialysis of the preparations to remove the ammonium sulfate failed to prevent the bitter flavor. The bitter flavor was attributed to proteolysis caused by residual trypsin in the xanthine oxidase preparation. An observation that indicated the presence of residual pro-

[1] Nutritional Biochemical Corp., Cleveland, Ohio. Prepared by the method of Ball (3), with crude trypsin as the proteolytic enzyme.

teolytic enzyme was that milk samples containing added xanthine oxidase showed definite proteolysis after the incubation in the xanthine oxidase determination (vanillic acid method). Because of interference of the bitter flavor in organoleptic examinations, the T.B.A. test (6) was used to measure oxidation. Aurand and Woods (1) also resorted to the use of this test.

Two of the impurities in the xanthine oxidase preparations would be expected to influence oxidized flavor with opposing effects. The residual proteolytic enzyme would be expected to inhibit development of oxidized flavor (7). The commercial preparation of xanthine oxidase contained copper at a level of 3 μg. per milliliter. Since 1-2 ml. of the xanthine oxidase preparation was required per 100 ml. of milk to increase the enzyme activity in some milks to that in samples high in activity, the copper content would have been increased as much as 0.06 μg. per gram of milk. This increase in copper would be expected to influence development of oxidized flavor in milks produced by the cows used in these trials (9).

The added xanthine oxidase was inconsistent in its effect on development of oxidized flavor as measured by the T.B.A. test, in some cases increasing, in others decreasing, the result in comparison with the control sample. Because of these inconsistencies and the uncontrolled variables described above, this experimental approach was considered unsatisfactory.

Determinations of xanthine oxidase and incidence of spontaneous oxidized flavor in individual milk samples did not support the observation of Aurand and Woods that there is a good correlation between xanthine oxidase activity and spontaneous development of oxidized flavor. The authors do not agree with the conclusion (2) that spontaneous oxidized flavor may be differentiated from nonspontaneous flavor on the basis of catalytic activity of xanthine oxidase. The results do not preclude the possibility that xanthine oxidase may be involved in catalysis of oxidized flavor, but in this study it was not a limiting factor.

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EFFECTS OF CONTINUOUS AND ALTERNATING STORAGE TEMPERATURES OF −79 AND −196° C. ON MOTILITY OF FROZEN BULL SEMEN [1]

Since the possibilities of frozen semen were noted (11), consideration has been given to bull semen stored in dry ice–alcohol (1,4,7,8), mechanical refrigeration (2,5) and, more recently, in liquefied gases at approximately −196° C. (3, 6, 9, 10). Motility studies have indicated that liquid nitrogen is a superior refrigerant as compared to dry ice–alcohol (3, 6, 9, 10). The purpose of this study was to further compare the effects on motility of continuous liquid nitrogen storage at approximately −196° C. to those of conventional dry ice–alcohol storage, approximately −79° C., and to determine the effects on motility of alternating these storage conditions. The latter objective could have considerable practical importance in the exchange of frozen semen between artificial breeding organizations employing different methods of storage.

EXPERIMENTAL PROCEDURE

This study was conducted utilizing semen collected from bulls routinely used at the Connecticut Artificial Breeding Association (CABA). A total of 17 ejaculates were obtained by an artificial vagina from four Guernsey, five Holstein, and three Jersey bulls. Immediately after collection, a hemocytometer count was made and the semen extended to contain, after glycerolation, approximately 30 million progressively motile sperm cells per milliliter of semen. The semen was extended to one-half the final volume at 32° C. and allowed to cool over a 3-hr. period to 5° C. The glycerol-containing fraction of the extender was added in four equal portions at 15-min. intervals. Final concentration of the extender was 20% egg yolk, 2.117% sodium citrate dihydrate, and 7% glycerol. Antibiotics were included at the rate of 1,000 units of penicillin, potassium G, and 1,000 γ of dihydrostreptomycin sulfate per milliliter of semen. After equilibration for 15 hr. at 5° C., the semen was hermetically sealed in 1.2-ml. glass ampules and frozen in dry ice–alcohol. The freezing rate used in this study was 1° C. per minute from 5 to −15° C. and 4° C. per minute from −15 to −79° C. (7). After freezing, the ampules from each ejaculate were divided equally and randomly, one-half being stored in dry ice–alcohol and the remaining one-half transferred to liquid nitrogen refrigerators (LNR-25-B) maintained at approximately −196° C. (13). Ampules were thawed in ice water and an aliquot of semen placed on a glass slide previously warmed to 32° C. for estimations of per cent progressively motile spermatozoa. Samples were viewed under phase-contrast illumination at 430 × magnification.

Portions from each ejaculate from each storage temperature were examined for per cent progressively motile sperm immediately post-freezing, and after 1, 2, 3, 4, 6, 8, 10, 12, 14, 16, and 18 wk. of storage. After 6 wk. of continuous storage at their respective temperatures, transfers of paired samples were made from liquid nitrogen to dry ice–alcohol, and from continuous dry ice–alcohol to liquid nitrogen. Estimations were made on these transferred samples at 2, 4, and 6 wk. post-transfer, and the remaining samples were then retransferred after 12 wk. to their original storage temperature for similar 2-, 4-, and 6-wk. evaluations.

RESULTS AND DISCUSSION

Motility results (Figure 1) showed a highly significant positive difference ($P<0.001$) in favor of the liquid nitrogen storage. The same level of significance was obtained in a previous study (9) over an 8-wk. storage period. It was, therefore, confirmed, under the conditions of these studies, that motility of semen stored continuously in liquid nitrogen is superior to semen stored continuously in dry ice–alcohol. The decline in motility of the semen stored at dry ice temperatures obtained in this study was expected, since reduction of motility of frozen semen during early weeks of storage has been reported previously; e.g., Buch *et al.* (2) ob-

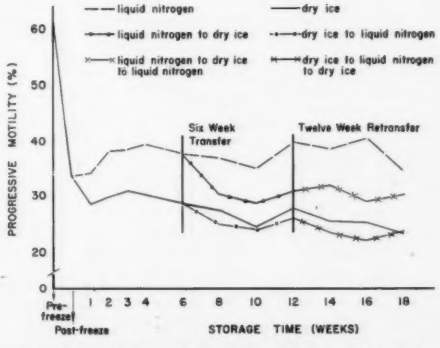

FIG. 1. Effects of continuous and alternating storage temperatures on motility of bovine spermatozoa. (Mean of 17 ejaculates.)

[1] Supported in part by grants-in-aid from Chas. H. Hood Dairy Foundation, National Association of Artificial Breeders, and New England Artificial Breeding Council. Portions of this paper were presented at the 33rd Annual Meeting of the Eastern Division of the American Dairy Science Association, September 21, 1959, at West Springfield, Massachusetts.

tained maximum decline in motility during the first 2 wk. of storage at −89° C., while O'Dell *et al.* (8), using milk, observed the greatest reduction in motility over 4 wk. of storage at −79° C.

Semen transferred from liquid nitrogen to dry ice–alcohol, after 6 wk. of storage, exhibited an immediate decline in motility similar to that of the original dry ice–alcohol stored semen. From motility observations on paired samples of this semen retransferred from −79° C. to its original storage temperature, it appeared that the decline in motility was permanent, since no appreciable increase in motility was observed after retransfer and subsequent storage for 6 wk. Additional evidence indicating permanent damage at −79° C. storage temperature was supported by the observation that motility of samples transferred from dry ice–alcohol to liquid nitrogen further declined, although not significantly (at $P = 0.05$).

Increases in average motility evaluations of frozen semen during storage, such as were seen in liquid nitrogen, have been observed by Etgen *et al.* (5). These authors postulated that some adjustment period is necessary for sperm to respond to the freezing and thawing processes, which may serve as an explanation of the results obtained with continuous liquid nitrogen storage. Also, Larson and Graham (6) found that semen transferred from −196 to −79° C., after three days of storage, showed a greater decline in motility than seven- or ten-day transfers. Thus, an aging period of at least a week was suggested by these investigators as necessary before transferring semen from temperatures of −196 to −79° C.

After semen had been stored in liquid nitrogen for 3 wk. or more, it appeared to the authors that the sperm cells moved more rapidly on thawing than those of the paired samples stored in dry ice–alcohol. If this observation is confirmed upon additional research, care will have to be taken to keep from over-evaluating samples from liquid nitrogen storage. This would probably happen to a greater extent in samples in which the dilution ratio was low, and might serve as a partial explanation in conjunction with an adjustment or aging period, for the increase in motility of samples stored in liquid nitrogen (Figure 1).

It can be seen (Figure 1) that average motility in continuous liquid nitrogen storage exceeded the original post-thaw estimations. This observation was not consistent with earlier reports from this station (9). Therefore, samples remaining from previous studies (9) were again evaluated at 13 mo. of age. For 12 of the original 17 samples available for study, a rather sharp increase of motility was noted in liquid nitrogen storage: 29.2 at 8 wk., as compared to 36.3 after 13 mo. of storage. The corresponding increase for the paired samples in dry ice–alcohol was from 17.5 to 18.9. It is recognized that the small increase in per cent progressive motility at dry ice temperature possibly could have been accounted for by estimation error and ampule difference. On the other hand, it seemed likely that the rather large increase in motility seen in liquid nitrogen storage samples could not be accounted for completely in this manner. Thus, it was assumed that a true increase in motility did occur.

Statistical analysis of the data in Figure 1 is shown in Table 1. Comparisons were made between motility means according to Snedecor (12). There was a highly significant difference between the means of seven of the nine comparisons. Continuous liquid nitrogen storage was superior to all other combinations to which it was compared. Semen stored in liquid nitrogen 6 wk. before it was transferred to −79° C. exhibited greater motility than any paired samples stored in dry ice originally, regardless of the latter transfers. It should be noted (Table 1) that semen stored in dry ice–alcohol con-

TABLE 1

Summary of "t" test comparisons between various treatments presented in Figure 1

Treatment		Mean motility			
I	II	I	II	Difference	Standard error[c]
		(%)			
LN[a] (cont.)	vs. DI[b] (cont.)	37.4	27.7	9.7	0.38*
LN (cont.)	vs. LN to DI	37.1	29.9	7.2	0.68*
DI (cont.)	vs. DI to LN	26.4	25.0	1.4	0.74**
LN to DI	vs. DI (cont.)	29.9	26.4	3.5	0.75*
LN to DI	vs. DI to LN	29.9	25.0	4.9	0.79*
LN to DI to LN	vs. DI (cont.)	30.0	25.7	4.3	0.92*
LN (cont.)	vs. LN to DI to LN	37.9	30.0	7.9	0.83*
DI (cont.)	vs. DI to LN to DI	25.7	23.0	2.7	0.93*
LN to DI to LN	vs. DI to LN to DI	30.0	23.0	7.0	0.63*

* = Significant at $P \leqslant 0.01$.
** = Significant at $P < 0.10 > 0.05$.
[a] = Liquid nitrogen storage.
[b] = Dry ice–alcohol storage.
[c] = Based on 16 degrees of freedom.

tinuously was better than its paired sample transferred to liquid nitrogen, and that retransfer to the original temperature was of little value.

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ERRATUM

O. W. Kaufmann *et al.* Relative Cleanability of Stainless Steel Finishes After Soiling with Inoculated Milk Solids. J. Dairy Sci., 43: 28. 1960.

The last sentence of the first paragraph on page 40 should be as follows: This test showed a difference between the No. 7 and the other finishes after T-2 with the Swab Test. The highest count was on No. 7.

INTERPRETIVE SUMMARIES OF PAPERS

STRONTIUM IN MILK. I. REMOVAL BY MEANS OF REVERSE-FLOW ION-EXCHANGE COLUMNS

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Since the first atomic bomb was detonated in 1945, the earth's atmosphere has contained radioactive material, commonly called fallout, which slowly settles to the earth. Weapons testing has tended to add to or to maintain this concentration of radioactive material in the air.

The dairy industry is particularly concerned about Strontium^{90}. This isotope will remain radioactive for many years and it acts chemically in a manner similar to calcium. Milk and dairy products supply about 80% of the calcium in the American diet.

Though the level of Sr^{90} in milk is very low, some citizens, public officials, and scientists are concerned about its being in milk. The object of this investigation was to learn how to remove the strontium and still maintain a palatable product.

Milk containing radioactive strontium was passed through a column containing ion-exchange resins, a product similar to that used in home water softeners. Some strontium in the milk replaced part of the calcium on the resin and some calcium on the resin took the place of strontium in the milk. Therefore, the milk contained less strontium and more calcium after it passed through the resin.

The per cent of strontium removed depended upon several conditions. These included source of milk, kind of resin, nature of resin-conditioning material, and the amount of milk exposed to a given quantity of resin.

Milk treated to remove strontium by this method showed higher acidities, lower pH levels, harder curd, and shorter rennet coagulation times than milk not so treated. The first milk coming through the column tasted slightly bitter, but the milk passing through later was nearly normal to the taste.

STRONTIUM IN MILK. II. REMOVAL BY BATCH ION-EXCHANGE METHODS

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The reverse-flow ion-exchange column for removing radioactive strontium from milk has already been investigated. The calcium-resin-column technique used did not directly permit the investigation of the effects of resin-milk contact time or the effects of various milk-resin ratios. Therefore, this study made use of the batch calcium-containing resin and the milk, mixed together in a container, then separated at the end of a specified time.

The data indicate that holding the calcium-based resin in contact with the milk for longer than 10 min. removes but little more strontium.

The spiking of the milk technique was studied, to see how long it takes strontium to mix with milk. The tests show that strontium will be as fully incorporated into the milk in 1 min. as it will in 60 min.

The amount of strontium removed from milk depends largely upon the milk-resin ratio used. These ranged from a high of 94% removal at a milk-resin ratio of two parts milk to one of resin, to a low of 21% at a milk-resin ratio of 200 to 1. The reverse-flow column technique was calculated to be about 86% as efficient as the batch system.

A METHOD FOR THE ELIMINATION OF ASHING IN STRONTIUM^{90} DETERMINATION OF MILK

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Ashing is a cumbersome first step in most procedures for the determination of strontium^{90} in milk. Introduction of rapid ashing techniques has facilitated this operation somewhat, but it is still time-consuming and requires special equipment to process liter-quantities of milk required for measurement of very low radioactivity levels.

Nonashing procedures for the analysis of milk for calcium, sodium, and potassium often involve great dilution of the original sample; whereas, for citrate analysis, milk is precipitated with trichloroacetic acid. Dilution is impractical for radionuclide analysis, but precipitation offers a promising alternative to ashing.

To explore the feasibility of trichloroacetic acid precipitation as a substitute for ashing, calcium and potassium determinations were made on milk and trichloroacetic acid–treated milk filtrates and were found to be essentially identical. When the alkaline earth elements (calcium, strontium, barium, etc.) were precipitated from the trichloroacetic acid filtrate, strontium was recovered quantitatively in the precipitate, thus demonstrating that the available strontium could be isolated from milk by this two-step procedure.

On the basis of these observations, the following method of preparing milk samples for strontium^{90} analysis has been devised: (a) Treat milk with an equal volume of 24% trichloroacetic acid, (b) remove fat and proteins by filtration and discard, (c) precipitate alkaline earth elements from the filtrate by adjusting to pH 8.5 to 9.0, and (d) proceed with analysis of precipitate for strontium^{90} by previously published methods.

Analysis of several milk samples prepared by both the precipitation and ashing methods yielded comparable strontium^{90} values and were shown to be not significantly different; therefore, the precipitation method of sample preparation may be used satisfactorily in the quantitative analysis of milk for strontium^{90}.

ROLE OF LEUCOCYTES IN THE REDUCTION OF RESAZURIN IN RAW MILK

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It is quite generally assumed that the ability of the resazurin test to detect mastitic milk is due to the reducing activities of leucocytes. Some workers have concluded that, at least with methylene blue, leucocytes do not contribute significantly to the reduction of the dye. The present work was an attempt to assess the role of the leucocytes of raw milk in the reduction of resazurin.

It was found that washed leucocytes, which had been freshly isolated from bovine blood, had a strong ability to reduce resazurin. At a concentration of 10^6 per milliliter they would bring about the reduction of the dye within 20 min. However, leucocytes isolated from milk had a definite but very weak ability to reduce the dye. A concentration of 4×10^6 per milliliter was required to increase the rate of reduction from 4 hr. to 3. This indicated that the reducing activity of the leucocytes in the udder was unstable and had decreased greatly. To test this possibility, washed leucocytes, which had been freshly isolated from bovine blood, were held in milk at 37° C. for 4 hr. before the addition of resazurin. This storage increased the reduction time from 20 min. to 3 hr. Even storage at 4° C. for 24 hr. increased reduction time from 20 to 110 min.

It is well known that reduction times for milks increase markedly on storage and one contributing factor is, undoubtedly, the instability of the reducing enzymes of leucocytes. When leucocytes, separated from raw milk, were suspended in fresh bovine plasma, they rapidly regained much of the reducing activity evident in leucocytes from bovine plasma. This regained activity could not be changed by repeated washing in saline. One might conclude that in a severely diseased udder, where plasma would be present, leucocytes would retain their reducing ability. However, in any other circumstances it would appear that the reducing activities of leucocytes do not contribute significantly to the reduction of resazurin in raw milk.

ULTRACENTRIFUGATION STUDIES OF MILK HEATED TO STERILIZATION TEMPERATURES

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The rate of sedimentation of suspended particles or large molecules in water solutions and the rate of rising of fat clusters in milk under the influence of gravity and the application of Stokes Law have been used by a number of investigators during the past 40 yr. Svedberg and Nicols[1] were the first workers to increase the force of gravity by centrifugation and thereby accelerate the sedimentation rate of molecules of different sizes. This technique has enabled

[1] Svedberg, T., and Nicols, J. B. Determination of Size and Distribution of Particles by Centrifugal Methods. J. Am. Chem. Soc., 45: 2910. 1923.

investigators to achieve forces of 300,000 times gravity. A common example of centrifugation is the collection of fat in the neck of Babcock test bottles in a centrifuge spinning at about 1,000 times the force of gravity. The ultracentrifuge, which is a high-speed centrifuge, is one of the best and most powerful tools available for investigating certain physical states of large protein molecules in raw and in heated milk.

The purpose of this investigation was to determine, immediately after heating and during storage, the physical stability of milk heated at increasing intensities by the ultra high-temperature, short-time method. Physical stability was determined by spinning milk in a high-speed centrifuge at 10,000 times gravity. Pictures were taken of the protein thrown down during centrifuging and its volume was measured.

Heating was done as follows: A. Milk was preheated at 180 ± 0.5° F. for 30 min., then in 2.08 sec. it was heated to 300 ± 0.5° F. and held for 2.08 sec., homogenized, and cooled. B. Same as A, except milk was sterilized at 285 ± 0.5° F. C. Milk was preheated to 180° F. in 2.08 sec., then heated to 300 ± 0.5° F. in 2.08 sec., and held for 2.08 sec., homogenized, and cooled. D. Same as C, except milk was sterilized at 285 ± 0.5° F.

The sedimented protein from raw milk was transparent. As the temperature of heating was increased, the amount of light transmitted through the sediment decreased. The sediment protein was opaque in milks heated above 310° F. with a 2.08-sec. hold and in milks which had been held at 180° F. for 30 min. before final sterilization. The presence of some fat in the sediment may have been responsible for this opacity. The maximum volume of sediment increased immediately after processing. Packing of the sediment was greater as the temperature of heating the milk was raised. The increase in volume of sediment, because of the addition of denatured serum protein to the sedimented casein with higher heat treatments, was greater than expected. This suggested changes in the shape of the protein particles, their structure, or hydration.

The sedimentation rates increased with the higher sterilization temperatures and the changes above the temperature needed for sterilization were greater than the differences between raw milk and the lowest sterilization temperature.

The volume of sedimented protein decreased during storage. It was less at 100 than it was at 70° F. This indicates that the larger protein particles decreased in size during storage. The volume of sediment was greatest at 40° F. storage, indicating that the proteins contained more water than they did at 70 and 100° F. storage.

A QUANTITATIVE TEST FOR CARRAGEENIN ESTER SULPHATE IN MILK PRODUCTS

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Carrageenin, or Irish Moss extractive, is widely used in the dairy industry as a stabilizer in various products, such as ice cream and chocolate drink. It is

extracted from the red sea-plant, *Chondrus crispus,* and consists of a mixture of high molecular weight sugar derivatives containing sulphate. The main components of this mixture are called kappa- and lambda-carrageenin. While qualitative tests for the various common vegetable gums in dairy products are available, a quantitative test has not been reported.

Since carrageenin is characterized by a high sulphate content, a test may conveniently be based upon this constituent. However, the usual methods for the determination of ester sulphate in carrageenin can not be applied to milk systems. Therefore, a modified procedure was developed which is suitable for dairy products.

One hundred grams of the dairy product to be tested is exhaustively dialyzed at 5° C. for 48 hr. by placing it in a Visking casing in distilled water. The water is changed five to six times during the dialysis procedure. Then the sample is transferred to a 400-ml. beaker, 10 ml. of concentrated hydrochloric acid is added, and the volume is made up to 200 ml. The beaker is covered and placed in a water bath at boiling temperature for 6 hr. to free the ester sulphate. While the sample is still in the bath, 8 g. of activated carbon and 2 g. of Filter Aid are added, to facilitate the removal of interfering substances. The sample is then filtered and washed with dilute hydrochloric acid (1:20). The filtrate and washings are combined and heated to 65–70° C. An excess of hot 5% barium chloride solution is added, and the solution is held at the same temperature for at least 6 hr. to allow for the precipitation of barium sulphate. The sample is then filtered through a weighed Gooch crucible packed with acid-washed asbestos. The Gooch crucible with the barium sulphate is heated to 900° C. in a muffle furnace, cooled to room temperature, and weighed. From the weight of the barium sulphate the amount of carrageenin ester sulphate can be calculated after correcting for the sulphate content of the reagents.

The method is accurate to 0.6 mg. % of sulphate and is suitable for routine analysis of dairy products. If the dialysis step is omitted, the same procedure may be used to determine the total sulphate content of the dairy product.

XANTHINE OXIDASE AND INCIDENCE OF SPONTANEOUS OXIDIZED FLAVOR IN MILK

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Oxidized flavor continues to be a troublesome defect in milk and certain dairy products. In spite of the voluminous literature on this defect, there is not yet agreement regarding the principal factors responsible for its spontaneous development. In recent publications, spontaneous oxidized flavor has been attributed to catalytic activity of xanthine oxidase, and also of copper. The purpose of this study was to determine if there is a relation between xanthine oxidase activity and the spontaneous development of oxidized flavor in milk.

Two methods were used to determine xanthine oxidase activity in milk from individual cows maintained on an alfalfa hay–grain concentrate ration. Milk

samples were examined for oxidized flavor after storage for two and five days. No relation was found between xanthine oxidase activity and the incidence of spontaneous oxidized flavor.

Since a recent report noted good correlation between xanthine oxidase activity and oxidized flavor development, several experiments were conducted in an attempt to explain the lack of agreement. The results did not explain the discrepancy.

It is concluded that spontaneous oxidized flavor can not be differentiated from nonspontaneous flavor on the basis of catalytic activity of xanthine oxidase. The results do not preclude the possibility that xanthine oxidase may be involved in catalysis of oxidized flavor, but in this study it was not a limiting factor.

THE AVAILABILITY AND METABOLISM OF VARIOUS SUBSTRATES IN RUMINANTS. I. ABSORPTION AND METABOLISM OF ACETATE. II. THE RATE OF ACETATE OXIDATION AS AFFECTED BY AVAILABILITY OF SUBSTRATE [1]

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Numerous studies have shown that the short-chain volatile fatty acids, acetic, propionic, and butyric, are the principal end-products of carbohydrate digestion in ruminants. Acetic acid is the predominant acid in the rumen and is the subject of the investigations reported here. After absorption from the rumen into the blood, acetate becomes available to the various tissues of the animal body. Biochemical studies have shown that acetate can participate in a number of metabolic reactions, including the fundamental energy-yielding reactions that result in its complete oxidation to carbon dioxide and water. This energy-furnishing role of acetate is of particular interest in ruminants because, unlike simple-stomached animals, little, if any, sugar is absorbed from the gut in these species.

Quantitative assessment of the role of acetate as an energy source in ruminants is a difficult problem and one for which suitable methods have not been available heretofore. It is to be expected that the amount of acetate available to the animal tissues will depend on the quantity and composition of the ration fed and the rate at which acetate is absorbed from the gut. The amount of absorbed acetate undergoing oxidation to yield energy may be expected to vary with the physiological and nutritional status of the animal. In the present investigations, techniques based on isotopically labeled acetate have been applied to the determination of acetate absorption rate and acetate oxidation rate in ruminants.

Carbon 14, a radioactive isotope of carbon, is a useful tool in biological research, because it can be incorporated into a number of naturally occurring substances, is easily measured, and does not alter the metabolism of substances into which it has been incorporated. In the present studies, acetate labeled with

[1] This is a summary of two papers in this Journal.

C^{14} was injected into the blood of dairy steers. Blood samples were taken periodically and the blood acetate assayed for radioactivity. Theoretically, the difference in specific activity (radioactivity/unit acetate) between the injected acetate and the blood acetate, after complete mixing of the injected labeled acetate with unlabeled acetate already present in the animal body, should provide a measure of the amount of acetate in the body. Furthermore, the rate of decline in the specific activity of the blood acetate provides a measure of the rate at which unlabeled acetate is entering the system. If the rate of acetate formation by tissues other than those of the gut is low, then the rate of decrease in specific activity of blood acetate is a function of the rate of acetate absorption from the gut.

The extent of acetate oxidation is determined from the amount of radioactivity recovered in expired carbon dioxide. The total amount of gas expired is measured and aliquot samples are analyzed for the amount of radioactivity of carbon dioxide present. A comparison of the amount of radioactivity recovered in expired carbon dioxide with the total amount injected provides a measure of the amount of acetate oxidized. The values for pool size (amount of acetate in animal's body other than the gut) and turnover rate (rate at which acetate enters and leaves the pool), obtained when a single injection of labeled acetate was administered to a mature dairy steer, were entirely unreasonable and indicated that this method of isotope administration was not applicable to metabolism studies with ruminants. In subsequent experiments, an initial injection of labeled acetate was followed by a continuous infusion of labeled material during the experiment. Using this technique, it was estimated that acetate was absorbed from the gut at the rate of 67 g/hour during the 3-hr. period after the steer was fed 4.3 lb. of a normal dairy grain mix. Approximately 50% of the absorbed acetate was oxidized to CO_2 and, since the amount of acetate in the pool did not increase, 50% was metabolized via pathways other than oxidation. It was further shown that approximately one-half of the total expired carbon dioxide came from acetate oxidation. If the assumption is made that the remaining carbon dioxide came from substrates with the same energy value as acetate, then acetate furnished approximately half of the energy utilized by the animal.

In the second paper, a series of experiments is described in which the rate of acetate oxidation was measured during the infusion of different amounts of labeled acetate. The rates of acetate oxidation were 15.5, 28.1, and 51.4 g/hour during acetate infusions of 20.3, 45.0, and 65.0 g/hour, respectively. The data show that as the amount of acetate oxidized to CO_2 increased, the amount of other substrates oxidized to CO_2 decreased. It is further shown that the maximum capacity of the steer to oxidize acetate had not been attained at the highest infusion rate, since the fraction of the infused dose recovered as CO_2 was the same at the highest and lowest rates of infusion. At the highest infusion rate, acetate oxidation supplied approximately 37% of the maintenance energy requirements.

THE EFFECT OF MILKING INTERVALS ON THE RATE OF MILK AND FAT SECRETION

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Early work in the field of milk secretion indicated that the rate of milk secretion (pound per hour) decreases as the internal pressure in the udder increases as a result of milk accumulation. This work is used to explain the increased production resulting from milking cows three and four times daily in comparison to twice-daily milking. This work is also the basis for the recommendation of milking cows at equal daily intervals.

More recent work from Australia, England, and New Zealand indicates that the milk secretion rate is constant for intervals of 18 to 24 hr. after the previous milking. Because of these conflicting reports, it seemed desirable to determine the effect of length of intervals on the rate of milk and fat secretion in high-producing cows.

Fifteen Holstein and Brown Swiss cows in early lactation were milked at 4-, 8-, 12-, 16-, and 20-hr. intervals. These cows averaged 64 lb. milk daily prior to the experiment. Each interval was repeated three times in succession and each cow was milked at all intervals. Oxytocin was injected after each milking to obtain the residual milk.

The results showed that the rate of milk, fat, and solids-not-fat secretion per hour was not changed for the 4-, 8-, and 12-hr. intervals. Significant reductions in all three components occurred at 16- and 20-hr. intervals. It was found that preceding long intervals had a deleterious effect on the rate of milk secretion during the following intervals. It was also found that the higher-producing cows in the group were less affected by the longer intervals than were the lower-producing cows.

By using the assumption that 20% of the milk in the udder at the beginning of milking remains in the udder after a normal milking procedure is completed, the expected rates of milk secretion for 10- and 14- and 8- and 16-hr. normal intervals were calculated. It was found that less than a 1% reduction in milk secretion rate would be expected at 10- and 14-hr. normal intervals and approximately a 4% reduction would occur using 8- and 16-hr. normal intervals, as compared to 12- and 12-hr. normal intervals. This work, along with other recent reports in the field, seriously questions the necessity of milking cows at exact 12- and 12-hr. intervals. Work is now under way at Cornell University to test these intervals for complete lactations.

THE NET ENERGY OF BLACKSTRAP MOLASSES FOR LACTATING DAIRY COWS

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Although cane or blackstrap molasses is often one of the cheapest sources of carbohydrates for animal feeding, there is lack of information on its pro-

ductive value when fed in varying amounts to dairy cows. It is commonly agreed that net energy (NE) is theoretically to be preferred as a measure of the productive value of feeds over such measures as total digestible nutrients (TDN) and digestible energy (DE). The classical method of determining NE, however, is such that the accumulation of information is slow and expensive.

The purpose of this study was to test a feeding trial method of determining NE, and to arrive at NE values for molasses when fed at levels of 10 and 30% of the total ration to lactating dairy cows.

The method of determining NE involved feeding a basal ration at two levels and replacing the basal with the two quantities of molasses. Energy yield was measured by determining the energy in freeze-dried milk solids. By feeding the basal at two levels, the increased energy yield brought about by the higher level represents the NE of the increase in basal consumed at this level. By measuring the energy yield when molasses replaces the basal, the NE of the molasses can be determined.

It was found that when blackstrap molasses replaced 10 and 30% of the basal ration, the NE values were 68.1 and 23.1 megacalories per 100 lb. of molasses, respectively. The point of this large loss of energy between 10 and 30% could not be determined in these studies.

Milk produced on 10% molasses in the ration could not be distinguished from that produced on the basal ration from the standpoint of taste, butterfat level, and rancidity scores. That produced by cows receiving 30% molasses, however, was of lower quality as measured by these tests.

On the basis of energy yield and milk quality tests this study indicates that 30% (8.5 lb. per cow per day) of molasses is too high for lactating dairy cows. Ten per cent, however, (3.1 lb. per cow per day) appeared to be used very efficiently.

THE EFFECT OF SEMINAL VESICULECTOMY ON CERTAIN PHYSICAL AND BIOCHEMICAL CHARACTERISTICS OF BOVINE SEMEN

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The essentiality of the secondary sexual glands has been questioned for many years. Fecundity, however, has been exhibited in cases where the seminal vesicle or prostate was surgically extirpated from animals that normally possess them. The functional activity of the bull's seminal vesicles may be only partially related to level of fertility.

This study was designed to investigate possible changes in the physical and biochemical characteristics of bull semen before and after the seminal vesicles had been surgically removed.

Four young bulls (one two-year-old Holstein-Angus, two yearling Jerseys, and one yearling Holstein) were used in the experiment. Physical evaluations

were made on ejaculate volume, spermatozoan concentration, pH level, per cent of motile spermatozoa, spermatozoan motility rate, and freezability. Biochemical assays were made on semen fructose, semen citric acid, and five seminal amino acids (aspartic acid, glutamic acid, serine, glycine, and alanine).

Semen was collected from the four bulls every five days during the study. Since a minimum of 5 ml. was necessary to conduct the series of tests, each bull was allowed to mount and ejaculate until the minimum volume was obtained.

The removal of the seminal vesicles resulted in a 50% reduction in the volume of semen per collection. With the decrease in volume of semen per ejaculate, a significant increase in spermatozoan concentration was noted in post-operative collections.

Following seminal vesiculectomy, increases in semen pH were noted. The mean pH increased significantly from 6.63, for the average of all bulls' collections before the operation, to 7.75 for postoperative collections.

When diluted semen was evaluated after zero, one, three, five, and seven days of storage at 5° C., the per cent of motile spermatozoa was significantly higher in the pre-operative collections. Spermatozoan motility rates were significantly higher before the operation when seven-day-old evaluations were compared. One-day-old evaluations of spermatozoan motility rates were not significantly different when pre- and post-operative collections were compared.

The freezability of bull semen was not significantly affected by the removal of the seminal vesicles. Possible detrimental effects, due to the absence of seminal plasma, may be delayed by the 24-hr. processing and freezing of the semen.

Seminal vesiculectomy resulted in severe changes in the amounts of semen fructose and citric acid. Analysis of pre- and post-operative collections revealed only traces of fructose and citric acid in the semen after the operation. These data stress the importance of the seminal vesicles as a source of the secretions rich in fructose and citric acid.

Chromatographic analysis of the amino acids of bull semen, before and after the removal of the seminal vesicles, resulted in no significant differences in the levels of the individual amino acids. In the pre-operative collections, the individual amino acid level of highest to lowest order of concentration was: glutamic acid, glycine, serine, alanine, and aspartic acid. In the postoperative collections, serine replaced alanine in the order of highest concentration.

An additional observation was made at the termination of the study. A lack of crest development and over-all masculinity was noted in the three younger bulls. Secondary sexual characteristics were well developed in the two-year-old bull prior to the operation and little change was noticed in this animal. These observations were made as long as 18 mo. following extirpation.

INFLUENCE OF YOLK-CITRATE, YOLK-GLYCINE, WHOLE MILK, AND SKIMMILK EXTENDERS CONTAINING GLYCEROL ON MOTILITY OF BOVINE SPERM AT 37 AND 5° C.

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Commercial artificial breeding associations use egg yolk–citrate or heated milk as the basic extenders when storing semen unfrozen. By the addition of glycerol, these extenders are suitable for freezing and storage of semen at temperatures below 75° C. Conventional extenders resist change in pH, have similar osmotic pressure to semen, give sperm protection against cold shock, and provide materials utilizable for sperm energy.

Our earlier studies have indicated that sperm do not survive equally well in the same extender when different storage conditions are used. The purpose of the present study was to compare several commonly used extenders and their mixtures, using two methods of adding glycerol and two storage temperatures (37 and 5° C.). Each of 16 semen samples were extended 1 : 50 in 14 different extenders. The basic extenders were egg yolk–2.9% sodium citrate, egg yolk–2.0% sodium citrate, whole milk, skimmilk, and egg yolk–glycine. Combinations of these were made by mixing equal parts of two basic diluters in all possible pair combinations.

Adding glycerol before extending the semen (direct) or doing it stepwise over a period of 160 min. after extending the semen had little effect on sperm motility. Though direct glycerolation was generally associated with higher progressive motility, the differences were not significant by seven days of storage at 5° C. in any of the extenders studied.

Egg yolk–2.9% sodium citrate and its combinations were superior at 37° C. storage, but were not as good as whole milk, skimmilk, or egg yolk–2.0% sodium citrate after seven days of storage at 5° C. Egg yolk–glycine in combination with either whole milk or skimmilk resulted in the highest average progressive motility after seven days of storage at 5° C.

No single basic extender or combination of two basic extenders gave similar results as time and temperature of storage and method of adding glycerol were varied. This experiment shows that extenders need to be developed and evaluated under the varied temperatures which the sperm will encounter in processing, storage, and insemination.

LACTIC DEHYDROGENASE ACTIVITY IN BOVINE SEMEN

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To formulate semen extenders that will prolong the longevity and fertilizing capacity of bovine semen, more basic information is needed concerning the en-

zyme activity of bovine semen. Previous research from various sources has established the fact that fructose is present in bovine semen and that there is an accumulation of lactic acid as the result of fructolysis. The presence of zinc in certain accessory glands and in the semen of the bull, and the presence of zinc in the molecule of lactic dehydrogenase, all point to the possibility of this enzyme being present in bovine semen.

This investigation was conducted to determine the lactic dehydrogenase activity in bovine semen and its relationship, if any, with 30–60 day nonreturn conception data, semen volume, and spermatozoa concentration.

The results indicate that the lactic dehydrogenase activity of bovine semen is high. There was a significant negative correlation between lactic dehydrogenase activity and the 30–60 day nonreturn conception data. Observations on 12 semen samples indicate that most of the lactic dehydrogenase activity is associated with the seminal plasma, which is composed chiefly of the secretions of the accessory glands, rather than the spermatozoa themselves. The average cell volume of spermatozoa in 12 semen samples having an average concentration of spermatozoa of 1,235,000 per cubic millimeter was 10.2%.

EFFECTS OF CONTINUOUS AND ALTERNATING STORAGE TEMPERATURES OF −79 AND −196° C. ON MOTILITY OF FROZEN BULL SEMEN

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The use of frozen semen has become commonplace with artificial breeding organizations. Some organizations are employing a full-time frozen semen program, while others are using frozen semen to supplement a liquid semen program.

The most common method of storing frozen bull semen is in dry ice and alcohol at a temperature of approximately −79° C. (−110° F.). Some organizations have used or are using mechanical refrigeration ranging from −84 to −95° C. (−120 to −140° F.) for bulk storage before transferring to dry ice units for field use.

Recently, there has been considerable interest in frozen semen storage in liquid nitrogen, a gas that when liquefied has a temperature of −196° C. (−320° F.). With the advent of equipment for both bulk and field storage of semen at this temperature, questions have arisen concerning the practicality of transferring frozen semen from dry ice and alcohol to liquid nitrogen, and vice versa.

The purpose of this investigation was to determine the effects on motility of bull semen stored at these two temperatures and of transferring portions of this semen to the colder, −196° C., or warmer, −79° C., temperature, depending upon the original storage method.

It was found that semen stored continuously in liquid nitrogen had greater motility than paired samples stored in dry ice–alcohol for 18 wk. Semen trans-

ferred from liquid nitrogen to dry ice and alcohol showed a sharp decline in motility, but did not drop below the semen stored continuously in dry ice.

Further observations on motility indicate that semen may also be transferred from dry ice–alcohol to liquid nitrogen, but that a small decline in motility could be expected.

QUANTITATIVE DETERMINATION OF FREE ESTRONE, ESTRADIOL 17-β, AND ESTRADIOL 17-α IN BOVINE FETAL COTYLEDONS

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Estrone, estradiol 17-β, estradiol 17-α, and estriol are collectively known as estrogens. These four have been frequently identified in tissues and fluids of several species of animals. Estrogens, probably estrone, and estradiol 17-β are required for normal reproduction. Such things as estrus, ovulation, preparation of the uterus for pregnancy, maintenance of pregnancy, mammary gland development, and development of femininity are some of the important functions.

Our knowledge of estrogen levels in cows is relatively meager, primarily because assay methods adaptable to cows were not available or too cumbersome for routine work. The primary purpose of the present study was to develop a chemical assay for estrogens in the fetal cotyledons of cow's placenta and, secondarily, to survey the levels of estrogens in the cotyledons at different stages of pregnancy.

A method for separating and measuring estrone and the two estradiols (17-β and 17-α) was developed. As little as 2 μg. can be detected in 300 g. of sample by this method. Other laboratories as well as our own have been unable to detect estriol in the cow. In contrast, estriol but not estradiol 17-α is found in the human. Both of these, especially estriol, have little apparent biological activity when tested in rats and mice and are probably metabolic by-products of estrone and estradiol 17-β.

During this study, estrogen levels were determined in 41 placentae. The total quantity of estrogens in placentae expelled normally after calving was from three to four times greater than the quantity present during the last 3 wk. of pregnancy, and 30 times greater than at 3–5 mo. of pregnancy. Before calving, estradiol 17-α was lowest and estradiol 17-β was highest. After calving, the reverse was true.

These findings suggest that normal pregnancy and normal calving in some way involve changes in estrogen levels in the placenta. These changes should be further evaluated in cases of late abortion, longer-than-expected gestations, and retained afterbirth.

OUR ASSOCIATION

EASTERN DIVISION MEETING
AMERICAN DAIRY SCIENCE ASSOCIATION

The 33rd Annual Meeting of the Eastern Division of the American Dairy Science Association was held September 21 in West Springfield, Massachusetts, at the offices of the Eastern Farmers' Exchange, Inc.

In a discussion on ways and means of improving the annual division meeting, it was decided to contact members in the Northeast to obtain their opinions. The alternatives presented were: 1. Investigate the possibilities of joining the meeting with the North Atlantic Section, American Society of Animal Production. 2. Develop a one-day meeting during the summer months to be held at a university campus in the area. 3. Continue with the present type of meeting, which is convenient for those with judging teams or for those attending the Eastern States Exposition. 4. Develop a one-day meeting during the week of Eastern States Exposition.

H. F. Judkins, Secretary-Treasurer of A.D.S.A., reported on the national program of A.D.S.A. and policies of the JOURNAL OF DAIRY SCIENCE. He strongly urged the divisional meetings to study and discuss the activities and policies of the National Association.

Dr. B. F. Trum, Director of the Animal Research Center, Harvard Medical School, discussed Atomic Energy and the Dairy Industry. He traced the pathway of radioactive elements from fall-out through the soil, plants, animals, and into the milk. He urged the Dairy Industry to develop a working plan to handle the milk supply in a given area in case of an industrial atomic accident.

The Intercollegiate Judging Contests in Dairy Cattle and Dairy Products drew a total of 17 teams—12 in Dairy Cattle and five in Dairy Products. At the annual Awards Banquet, the University of Maryland was announced as the winner in Dairy Cattle, and the University of Connecticut won the Dairy Products Contest.

The officers elected were: President: B. R. Poulton, Department of Animal Industry, University of Maine, Orono; Vice-President: F. E. Potter, Department of Dairy and Animal Science, University of Massachusetts, Amherst; Secretary-Treasurer: H. A. Keener, Department of Dairy Husbandry, University of New Hampshire, Durham.

ABSTRACTS OF PAPERS
PRESENTED AT THE EASTERN DIVISION MEETING

WEST SPRINGFIELD, MASSACHUSETTS, SEPTEMBER 21, 1959

Grass-legume silage as the sole forage for feeding dairy cattle during the summer. B. W. HENDERSON, JR., AND J. W. COBBLE, University of Rhode Island, Kingston.

A comparison was made between grass-legume silage and soilage as the sole forage for 24 lactating dairy cows during the summer season. The cows were paired in relation to breed, age, size, and production of 4% fat-corrected milk.

In a 16-wk. trial in 1958, the cows receiving silage had an average FCM production of 36.3 lb., compared to 35.9 lb. for the soilage group. The dry matter intakes for the silage and soilage groups were 22.2 and 31.7 lb., respectively. The soilage group had a higher dry matter intake during the last 8 wk. of the trial.

The silage group maintained a more constant body weight, showing a net gain of 5 lb. per cow during the feeding trial. The soilage cows lost weight during the first 8 wk., but showed rapid gains during the last half of the summer which apparently were related to the high dry matter intake. The average gain of this group was 46 lb. per cow.

During a 10-wk. test period in 1959, average FCM production was 36.0 and 41.1 lb., respectively, for silage and soilage. Daily dry matter intake was 21.0 lb. for the silage group and 30.4 lb. for the soilage group. Body weight changes for the two groups were not significantly different.

Digestibility of certain hay types when supplemented with 16% grain. J. G. ARCHIBALD, H. FENNER, AND D. F. OWEN, JR., University of Massachusetts, Amherst.

Two hay types (legume-grass and mixed grass) were supplemented during the past winter season with a mixed concentrate (16%). Eight individual digestion trials of 20 days' duration were run, using four cows on each hay type. The ratio technique was used with chromium trioxide (Cr_2O_3) as the indicator.

The grass hay had significantly higher digestibility of dry matter, energy, and fiber, the values for fiber being especially consistent.

Comparison with results of similar trials for three earlier years (1955-58), when the hays were fed without any grain, show that the grain supplement increased the average digestibility of both rations, the increase being somewhat higher for the grass hays. Digestibility of each of the several nutrients determined (protein, fiber, N-free extract, and energy) was also markedly increased, and the significance of the difference in digestibility between the two hays was enhanced for dry matter, fiber, and energy.

Milk production on the grass hay on a par with the legume-grass combination over a 4-yr. period, appears to be due in part to significantly higher digestibility of the fiber in the grass hay. The grain supplement appears to have benefited the grass hay more than it did the legume-grass combination.

Further investigations on the value of pelleting the concentrate portion of the lactating cow's ration. H. P. Adams and R. E. Ward, Eastern States Farmers' Exchange, West Springfield, Massachusetts.

Previous investigations have indicated that a concentrate pelleted in ½-in. pellets will lower butterfat test significantly without affecting milk production. This reports the results of two trials in which a mash concentrate is compared to one in 5/32-in. pellets. Each trial was conducted with 12 cows divided into two groups for a double-reversal trial. The first trial consisted of from 3- to 60-day periods and the second from 3- to 57-day periods. During both trials, the forage consisting of hay, grass silage, and corn silage was weighed and fed in sufficient quantities to insure weighbacks. Concentrate feeding was adjusted twice monthly, based on FCM production. Milk was weighed at each milking and samples were taken for butterfat testing twice monthly.

The results of the first trial showed a significant difference in milk production in favor of the pelleted concentrate. FCM and butterfat production were also higher (not significant) on the pelleted concentrate. Butterfat test was higher (not significant) in favor of the mash concentrate.

The results of the second trial showed a small, insignificant difference in milk production in favor of pellets. However, butterfat production was significantly higher and butterfat test was highly significantly higher on the mash concentrate. FCM was higher (not significant) on the mash concentrate, also.

The results of four trials show no consistent effect on milk production, but in each trial the butterfat test was higher when the mash concentrate was fed.

Effects of continuous and alternating storage temperatures on motility of frozen bull semen. A. K. Fowler, B. W. Pickett, and W. A. Cowan, University of Connecticut, Storrs.

Preliminary evidence has shown that motility of frozen bull semen stored in liquid nitrogen (−196°C.) is superior to split-ejaculates stored in dry ice–alcohol (−79°C.). The purpose of this study was to determine the motility differences associated with alternating these storage conditions. Seventeen ejaculates from twelve bulls were frozen and divided equally for storage in dry ice–alcohol and liquid nitrogen. Portions of each ejaculate from each storage temperature were thawed and estimated for per cent progressive motility immediately after freezing and after 1, 2, 3, 4, 6, 8, 10, 12, 14, 16, and 18 wk. of storage. Transfers of paired samples were made from one storage temperature to the other after 6 wk. of continuous storage at their respective temperatures. Following motility estimations at 2, 4, and 6 wk. post-transfer, the remaining samples were retransferred to their original storage temperature for a similar 2-, 4-, and 6-wk. evaluation.

A highly significant difference in motility ($P<0.001$) was found between continuous liquid nitrogen and dry ice–alcohol storage in favor of the colder temperature. Semen transferred from −196 to −79° C. showed an immediate decline in motility similar to that observed during the initial 2-wk. dry ice–alcohol storage period. Motility of semen transferred from −79 to −196° C. fell slightly below the motility of continuous dry ice–alcohol storage. Slight motility declines were noted in both retransfers to the original storage temperature.

Corn silage versus mixed hay as roughage for Holstein calves. E. M. Kesler, J. M. Wilson, and W. H. Cloninger, Pennsylvania State University, University Park.

Three groups of 12 Holstein calves each were raised from birth to 20 wk. of age on ad libitum feeding of roughages as follows: Group I, fed second-cutting alfalfa-grass hay which had been field-cured without rain damage; Group II, corn silage harvested in late-milk stage of maturity; Group III, free choice of both roughages. Each calf was fed 60 lb. whole milk, including colostrum, and 39.4 lb. dry milk replacer (in water) during the first 6 wk. A simple 20% protein calf starter was fed ad libitum to a maximum of 3 lb. daily. Gains in body weight averaged 209, 220, and 223 lb. for Groups I, II, and III, respectively. Gains in chest circumference averaged 13.5, 13.8, and 14.0 in., and gains in height at withers, 20.0, 20.4, and 20.6 cm. None of these differences was statistically significant. Starter consumptions by calves in the three groups averaged 331, 341, and 329 lb., respectively. Group I consumed an average of 431 lb. hay per calf; Group II ate an average of 1,244 lb. silage (22% dry matter); Group III averaged 77 lb. hay and 1,255 lb. silage. It is concluded that good-quality corn silage is a satisfactory roughage for young Holstein calves.

Spontaneous and induced lipolysis in milk during switchback feeding of alfalfa hay and beet pulp. R. G. JENSEN, G. W. GANDER, AND A. H. DUTHIE, University of Connecticut, Storrs.

Two groups of Holsteins, five in each group, were alternately fed alfalfa hay or beet pulp in addition to an adequate ration of grain and grass silage. These feeds were switched once a month for 3 mo. Milk samples were taken once a week and analyzed by the silica-gel method for initial acid degree value, acid degree value due to spontaneous lipolysis, and acid degree value due to induced lipolysis.

Analysis of variance and switchback analysis indicated that the change of feeds had no influence on initial acid degree value, spontaneous lipolysis, or induced lipolysis. The analytical error was smaller than either the cow or feed error.

Freezing point observations of vacuum-treated milk. J. M. MOORE AND A. C. SMITH, Storrs (Connecticut) Agricultural Experiment Station.

The vacuum treatment of fluid milk by non-steam-injection flavor removal equipment resulted in an elevation of the freezing point at low levels and a depression at high levels of flash-cooling when processing homogenized milk at 172° F. in various adaptations of a Vacu-Therm unit. Four replicates with three or four different degrees of flash-cooling increments were run on each adaptation. The freezing point was determined by means of a Fiske cryoscope. The elevation or depression of the freezing point was represented by the difference between the freezing points of the raw and treated milk samples.

The downward trend of freezing-point differences, when plotted against degrees of treatment, expressed as per cent concentration, was represented in the following equations, where Y denotes freezing point elevation or depression, and X the per cent concentration: (1) $Y = 0.005 - 0.006\ X$ (single vacuum chamber before final heating); $Y = 0.006 - 0.015\ X + 0.004\ X^2$ (single vacuum chamber before final heating with a vapor line condenser); (3) $Y = 0.003 - 0.005\ X$ (single vacuum chamber after final heating); and (4) $Y = 0.008 - 0.014\ X + 0.002\ X^2$ (double vacuum chamber).

A simplified cleaning-in-place system. J. E. BEAM, University of Massachusetts, Amherst.

Since a large fraction of the labor in food plants is expended with the cleaning and sanitizing of processing equipment, a project was initiated at the University of Massachusetts to design and install a simplified cleaning-in-place system (C.I.P.) control capable of cleaning, automatically, a high-temperature, short-time (H.T.S.T.) pasteurizer, a homogenizer, and the sanitary piping.

An inexpensive multi-cam repeat-cycle timing device was used to program the cleansing cycle. The Taylor H.T.S.T. controller installed as part of the H.T.S.T. pasteurizer was utilized to regulate solution temperatures and record the cleaning sequence. Installation costs were further reduced by having the hot water circulating unit of the pasteurizer in the cleaning operation. This unit served as the make-up tank and circulation pump. Solution levels and pump operation were controlled through the use of liquid-level probes inserted into the make-up tank.

Four commercial detergents were evaluated for 9 mo. to determine their adaptability to automatic C.I.P. cleaning. The study showed similar results from a bacteriological standpoint, but wide variation in the solubility of alkaline detergents and the ability to prevent film formation on the surfaces of equipment. At no time during this period was the homogenizer successfully cleaned. The C.I.P. control proved to be dependable and trouble-free during 9 mo. of operation.

BOOK REVIEWS

METHODS OF BIOCHEMICAL ANALYSIS. Vol. 7. Ed. by David Glick. Interscience Publishers, Inc., New York. 1959. 353 pp. $9.50.

This seventh volume in the annual series continues to reflect the selection of topics emphasizing developments and innovations of both continuing and current interest. The articles included are the following: Immunoelectrophoretic Analysis, by Pierr Grabar; The Analysis of Basic Nitrogenous Compounds of Toxicological Importance, by A. S. Curry; Spectrophotometry of Translucent Biological Materials—Opal Glass Transmission Method, by Kazuo Shibata; The Determination of Inositol, Ethanolamine, and Serine in Lipides, by John M. McKibbin; The Assay of Lipoprotein Lipase in Vivo and in Vitro, by Edward D. Korn; Determination of Creatinine and Related Guanidinium Compounds, by John F. Van Pilsum; The Determination of Ethyl Alcohol in Blood and Tissues, by Frank Lundquist; and Determination of Heparin, by Louis B. Jaques and Helen J. Bell, followed by an author and subject index to Volume 7 and a cumulative author and subject index to Volumes 1 through 7.

It is the reviewer's opinion that the usefulness of each succeeding volume in this series decreases somewhat. This is true also as far as gross content is concerned, since each volume tends to get succeedingly shorter in total pages. One gains the impression that the accumulation of first-rate articles for this series is becoming an increasingly difficult job for the editor.

On the positive side, the articles present in this volume, by and large, continue to maintain the high excellence of coverage provided in the articles in previous volumes. Anyone interested in specific analyses of the materials covered will find the volume to be invaluable and, for this reason, as with previous volumes in the series, Volume 7 belongs on the bookshelf of the chemist concerned with a complete repertoire of biochemical techniques.

B. L. LARSON
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PRINCIPLES OF DAIRY CHEMISTRY, Robert Jenness and Stuart Patton. John Wiley and Sons, Inc., N. Y. 446 pp. 1959.

In the preface of the book the authors state: "We and many of our associates in American agriculture colleges long have felt the need for a textbook designed for teaching the principles of dairy chemistry. This book represents an attempt to fill that need. We have not had in mind a high-level reference textbook or a manual of methods of testing and analysis, but rather an interpretation and integration of widely scattered data in the dairy field, with basic principles of chemistry. We have striven to produce a book suitable for instruction of the undergraduate student of dairy science. The material should be easily handled by a student who has had one year each of inorganic and organic chemistry and a semester or quarter of biochemistry."

This stated objective has been achieved with considerable success. The book is definitely aimed at the advanced undergraduate who possesses a fair background in chemistry. A rather thorough discussion is given those areas in which our knowledge is reasonably well established. The student will benefit particularly from the numerous instances in which very logical interpretations of facts and theories are presented.

The subject matter is treated in chapters entitled The Composition of Milk, Milk Lipides, Lactose, Milk Proteins, Milk Salts, Milk Enzymes, Miscellaneous Substances, Physical Properties of Milk, The Physical Chemistry of Milk Fat Globules, Physical Chemistry of the Caseinate-Phosphate Particles in Milk, Effects of Heat on Milk, Flavors and Off-Flavors in Milk and Its Products, and Nutritive Value of Milk. Two appendices deal with recommended dietary allowances and a summary of information on the vitamins.

The book is considered a useful addition to the library of any dairy or food technologist.

JOSEPH TOBIAS
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LEHRBUCH DER RINDERBESAMUNG. Grundlagen, Technik, Organisation und züchterische Probleme der Samenübertragung beim Rind. (Textbook on the Insemination of Cattle. Principles, Technique, Organization, and Breeding Problems of Insemination in Cattle). K. Eibl. Paul Parey, Berlin and Hamburg. 485 pp. 1959.

This book, written in German and based on the author's interest and several years of experience, treats the practical aspects of artificial insemination rather completely. The first section of the book is devoted to the genital tract of the bull, and to semen and its collection, examination, dilution, and preservation. Part Two deals with the anatomy and functioning of the reproductive organs of the cow, insemination procedures, and fertility results. In the third section, the role of artificial insemination in cattle breeding is covered from the historical standpoint, from the standpoint of its advantages and disadvantages, in connection with bull selection and proving, and in relation to the necessary governmental controls. Part Four deals with the establishment and organization of artificial insemination stations. The last two sections are devoted to the daily routines involved in the collection, laboratory processing, and shipping of semen.

The author has done an excellent job of covering the broad aspects for managers and employees of artificial breeding organizations under European conditions.

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